Modulation of the gastrointestinal barrier function by probiotic *Escherichia coli* Nissle 1917

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr.rer.nat.)

genehmigte
D i s s e r t a t i o n

von Sya Nomna Ukena
aus Aurich

1. Referent: Professor Dr. Jürgen Wehland
2. Referentin: Professorin Dr. Petra Dersch
eingereicht am: 10.04.2006
mündliche Prüfung (Disputation) am: 13.07.2006

Voreröffentlichungen der Dissertation

Teilergebnisse aus der vorliegenden Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in nachfolgenden Beiträgen vorab veröffentlicht:

Publikationen

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Coldewey S., Suerbaum S., Buer J. and Gunzer F. The host response to the probiotic *Escherichia coli* strain Nissle 1917: Specific up-regulation of the proinflammatory chemokine MCP-1. BMC Med Genet. 2005, Dec 13;6(1):43.

Tagungsbeiträge

Ukena SN., Westendorf AM., Hansen W., Geffers R., Toepfer T, Rohde M., Buer J. und Gunzer F. Genexpressionsanalyse von Caco-2 Zellen kultiviert mit O157 und non-O157 EHEC, *E. coli* Nissle 1917 und *S. boulardii*: Gibt es probiotika- und pathogenspezifische Regulationsmuster? EHEC Workshop, Wildbad Kreuth, 2004

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Toepfer T, Buer J. und Gunzer F. *In vitro* and *in vivo* analysis of the probiotic-host interaction between *E. coli* Nissle 1917 and intestinal epithelial cells. 56th Annual Meeting of the DGHM, Münster, 2004.

Ukena SN., Gunzer F., Suerbaum S., Buer J., Westendorf AM. Probiotic *Escherichia coli* strain Nissle 1917 enhances the epithelial barrier function through modulation of ZO-1 expression. 57th Annual Meeting of the DGHM, Göttingen, 2005.

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Coldewey S., Suerbaum S., Buer J., Gunzer F. The impact of probiotic *Escherichia coli* strain Nissle 1917 on intestinal epithelial cells: Specific up-regulation of the proinflammatory chemokine MCP-1. 57th Annual Meeting of the DGHM, Göttingen, 2005.

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Suerbaum S., Buer J., Gunzer F. Probiotic *E. coli* Nissle 1917 specifically upregulates MCP-1 expression in intestinal epithelial cells: Contradiction to its probiotic nature? International Symposium Inflammatory Bowel Diseases – Research Drives Clinics, Münster, 2005.

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Suerbaum S., Buer J., Gunzer F. The host response to probiotic *Escherichia coli* strain Nissle 1917: Analysis of the gene expression in intestinal epithelial cells. 36th Annual Meeting of the German and Scandinavian Societies of Immunology, Kiel, 2005.

Ukena SN., Prettin S., Gunzer F., Suerbaum S., Buer J., Westendorf AM.
The impact of probiotic *Escherichia coli* strain Nissle 1917 on intestinal epithelial cells: up-regulation of ZO-1 expression. Falk Symposium 153 Immunoregulation in Inflammatory Bowel Diseases – Current Understanding and Innovation, Intestinal Disease Meeting, Berlin, 2006

Ukena SN., Westendorf AM., Hansen W., Rohde M., Geffers R., Coldewey S., Suerbaum S., Buer J., Gunzer F. The host response to the probiotic Escherichia coli strain Nissle 1917: specific up-regulation of the proinflammatory chemokine MCP-1. ENII-Mugen Immunology Summer School May, Sardegna, 2006.

Ukena SN., Westendorf AM., Hansen W., Suerbaum S., Buer J., Gunzer F.
E. coli Nissle 1917 induces a proinflammatory host response – contradiction to its probiotic nature? 16th European Congress of Immunology, Paris, 2006.

Weitere Publikationen

Goelden U., Ukena SN., Pfoertner S., Hofmann R., Buer J., Schrader AJ. RAR-beta(1) overexpression in chromophobe renal cell carcinoma: a novel target for therapeutic intervention? Exp Oncol. 2005 Sep;27(3):220-224.

Pfoertner S., Goelden U., Hansen W., Ukena SN., Toepfer T., Knobloch R., Hofmann R., Buer J., Schrader AJ. Cellular Retinoic Acid Binding Protein 1: Expression and functional influence in renal cell carcinoma. Tumor Biology 2005;26:313-323.

Weitere Beiträge

Ukena SN. Lactobacillen in der Pouchitis-Therapie, Yakult Newsletter 06/2005.

Ukena SN. Mechanismen des probiotischen Effekts, Yakult Newsletter 06/2005.

Ukena SN. Einfluss der Darmflora auf das Immunsystem, Yakult Newsletter 01/2006.

CHAPTER I - Introduction -

CHAPTER V - Supplement -

CHAPTER VI - References -

CHAPTER I

Introduction

Interaction of microbes with the host intestinal epithelium

The human gastrointestinal tract, home to great numbers and a vast diversity of microbial life, maintains complex physical and chemical barriers that allow normal physiological functioning amidst a large and complex bacterial community. The microbes of the healthy human intestine certainly provide some benefit, particularly by generating important metabolites and by reducing the colonization efficiency of dietary pathogens via occupying their potential niches. Probiotics play a relevant role in this context as their beneficial activities most likely result from complex interactions of the microorganisms with the intestinal microflora and the gut epithelium of the individual (Marteau *et al.*, 2001).

However, certain bacteria - pathogenic as well as non-pathogenic - have evolved a variety of mechanisms to breach the host barriers and gain systemic access. This is usually avoided by the innate and the adaptive immune system, which use specialized cells to fight off invading microorganisms.

The epithelial cells that cover the gastrointestinal tract are the front line of defense against the diverse populations of commensal and pathogenic microbes that thrive within the lumen of the intestine (Hooper *et al.*, 1998). The intestine's primary protection from the luminal flora is the highly selective barrier - specifically intercellular tight junctions - formed by the intestinal epithelial cells. Disruption of the integrity of this intestinal epithelial barrier by pathogenic microbes and their metabolites alters paracellular permeability and is a key feature of intestinal bowel diseases. The role of a 'leaky gut' in the pathogenesis of gastrointestinal diseases is of increased interest and the use of probiotics as potential therapeutic agents in gastrointestinal diseases is promising.

I. Introduction

1. Structure and function of the gastrointestinal tract

The human gastrointestinal tract is classified into a upper and lower part. The upper gastrointestinal tract begins with the mouth and is followed by the pharynx, esophagus and stomach (fig. 1). The lower gastrointestinal tract comprises amongst others the small intestine, 6 m in length and, along this length, is divided into three regions: the duodenum, the jejunum, and the ileum. These regions are functionally distinctive, although there is some overlap.

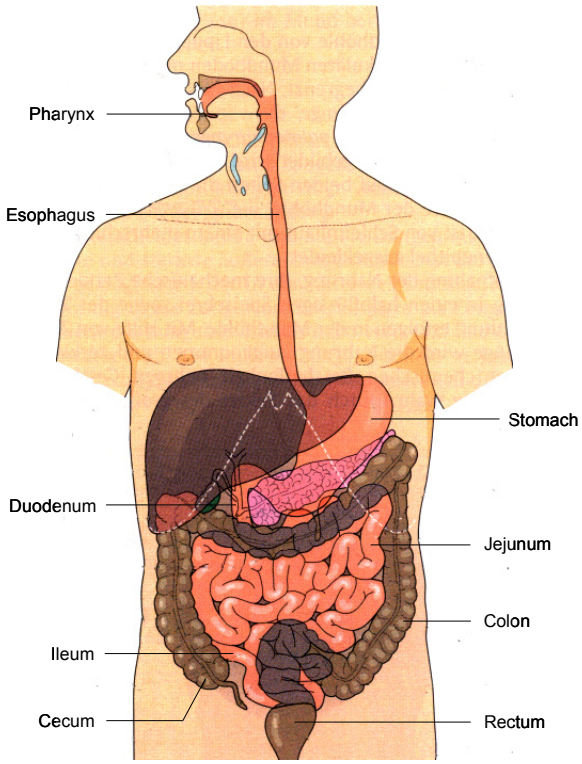

Figure 1. Schematic presentation of the human gastrointestinal tract.
The gastrointestinal tract is composed of an upper part with the mouth, pharynx, esophagus and stomach and a lower part. The latter comprises the small intestine (divided into duodenum, jejunum and ileum), the large intestine (divided into cecum, colon, rectum) and the anus. (adapted from Faller, 1995)

For example, brush border digestive enzyme function is important in the duodenum and jejunum. The jejunum is the principal site of absorption for Na^+ cotransport of monosaccharides, amino acids and uptake of fatty acids. The duodenum and jejunum are also the primary absortive sites for water-soluble vitamins, iron, and calcium. In contrast, bile salts and vitamin B_{12} are absorbed in the ileum. The lower gastrointestinal tract is followed by the large intestine, with the cecum, colon and rectum. The colon, approximately 1 m in length, is mainly responsible for absorption of Na^+ and Cl^- ions, water and secretion of K^+ and HCO_3^- ions.

Besides, these primary functions of digestion and absorption of various essential nutrients, a more attentive analysis of the anatomic and functional arrangement of the gastrointestinal tract reveals that another pivotal function of this organ is its ability to regulate the trafficking of macromolecules between the environment and the host and to prevent entrance of microorganisms into epithelial cells through a barrier mechanism. The total mucosal surface in the adult human gastrointestinal tract extends to 200-300 m^2, the largest area of the body in contact with the external environment.

2. Functional morphology of the intestinal mucosa

2.1 Mucosal structure

The intestinal mucosa consists of a one-cell-thick layer, the mucosal epithelium, directed to the lumen and composed of several diverse cell types. Located below the mucosal epithelium is a supporting layer of stroma, the lamina propria. The lamina propria harbors various immunocompetent cells including dendritic cells, macrophages, and lymphocytes, which form a functional unit with the mucosal epithelial cells. In addition, the mucosa is composed of a layer called submucosa and a delicate layer of smooth muscle, the muscularis mucosae (fig. 2).

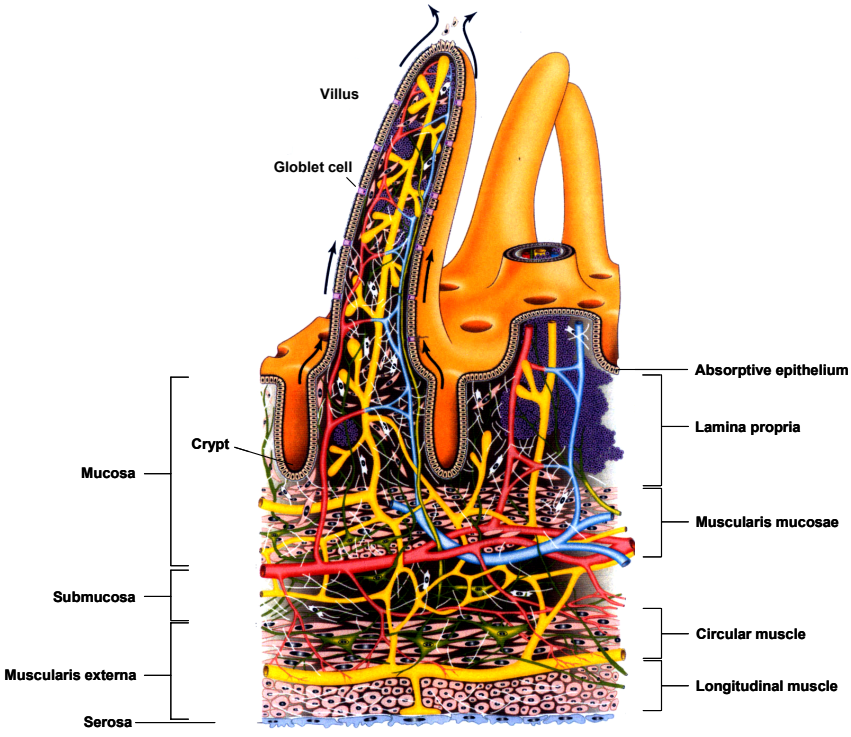

Figure 2. A 3D-reconstruction of the architecture of an intestinal villus. Also shown are arteries and arterioles (red), veins and venules (blue), central lacteals and other lymphatic channels (yellow), lymphoid follicles (purple), neural elements (green), smooth muscle fibres (pink), and fibroblasts (white). Arrows indicate the direction of cell migration. (adapted from Standring, 2005)

Although not part of the epithelium, mucus on the surface of the mucosa shields the mucosal epithelial cells from direct contact with the intestinal luminal environment. Furthermore, the mucus, produced by secreted mucins as a thick layer along the intestinal membrane and composed of large-molecular-weight glycoproteins, has several protective functions. The mucus forms a viscous blanket, that contributes to the prevention of penetration of microorganisms. The carbohydrate moieties that constitute the majority of the mucus structure can act as competitive binding sites – many infectious agents adhere to epithelial cells through cell-surface appendages that have carbohydrate binding properties (Freter, 1981). The release of mucus also

acts as a barrier by generating a stream that draws luminal contents away from epithelial cells.

In the small intestine, both the mucosa and underlying submucosa are specialized to increase the absorptive surface area. The submucosa is organized into regular ridges, which have been estimated to increase the available surface area 3-fold. Within the mucosa, the lamina propria is arranged into the supporting structure for villi.

2.2 The mucosal epithelium

The mucosal epithelium is mainly constituted of absorptive epithelial cells (enterocytes) - which are about 25 µm in height, 8 µm in width, and columnar in shape (fig. 3). Their apical surface has numerous tightly packed microvilli, increasing the surface area by an additional 10-fold. Villus enterocytes form a brush border and are highly specialized for nutrient digestion and absorption. Therefore, the apical surface of these cells is covered with a glycocalyx, mainly composed of carbohydrates enchored into the surface of the microvilli, containing various enzymes and nonenzymatic proteins. In contrast to the villi, the crypts are responsible for cell renewal, ion and water secretion, exocrine secretion of macromolecules into the crypt lumen, and endocrine/paracrine secretions into capillaries and the lamina propria.

At their apices, the enterocytes are connected with adjacent epithelial cells mainly by junctional complexes consisting of three major components: tight junctions (zonula occludens), adherens junctions (zonula adherens), and desmosomes (macula adherens). In addition to the junctional complexes, the lateral membranes interact by means of cell adhesion molecules, gap junctions, and interdigitations (Boyer and Thiery, 1989).

The structural integrity of each microvillus is maintained by a cytoskeletal core composed of actin filaments and associated proteins such as myosin I and villin. As these cytoskeletal cores enter the body of the cell, they encounter a dense mesh of microfilaments, marked as terminal web.

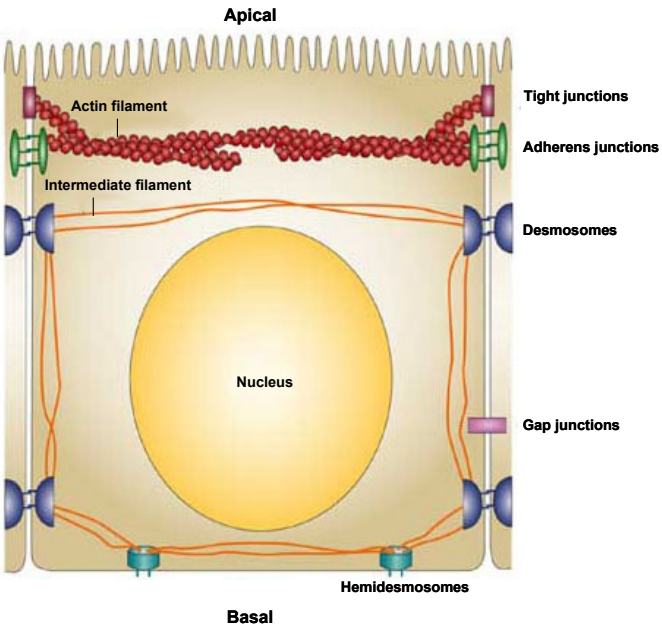

Figure 3. Schematic presentation of a polarized absorptive epithelial cell. The different types of intercellular junctions, as well as hemidesmosomes, a type of cell-extracellular matrix adhesion, are shown. It should be noted that tight junctions and adherens junctions are linked to the actin cytoskeleton, and desmosomes and hemidesmosomes are linked to intermediate filaments. (adapted from Matter and Balda, 2003)

Undifferentiated cells are derived from the epithelial stem cells, pluripotent epithelial cells located several cells above the base of the crypt or at the crypt base, and produce daughter cells that continuously migrate upward to the crypt apex and downward to the crypt base (fig. 2). These cells differentiate into enterocytes, the mucin-producing globlet cells, Paneth cells and enteroendocrine cells. One characteristic feature of Paneth cells is the secretion of proteins like lysozyme (Vantrappen and Peters, 1974), tumor necrosis factor (Schmauder-Chock *et al.*, 1994) and cryptdin (Ouellette and Lualdi, 1990). Therefore the primary function of Paneth cells may be the elaboration of antimicrobial peptides (Ayabe *et al.*, 2004). Consistent with this is the observation that Paneth cell numbers may increase in relation to luminal bacterial load (Elmes *et al.*, 1984).

2.3 Intestinal epithelial cells function as a physical barrier

The gastrointestinal tract encounters an enormous load of antigens. Although most of these antigens are beneficial (e.g. symbiotic bacteria) or harmless (e.g. commensals) to the host, it is mandatory to recognize harmful antigens and inhibit them from penetration. Since the gastrointestinal tract is mostly covered only by a single-layer epithelium – in contrast to e.g. skin – it requires a more extensive protection against antigen access. Once antigens have negotiated the mucus layer and evaded proteolysis, there is another considerable barrier formed by the intestinal epithelial cells (IECs). This physical (intrinsic) barrier consists of two main components: a layer of interconnected, polarized epithelial cells (transcellular route) separated by a basal

membrane from the connective and supporting tissue and the tight junctions present in the spaces between cells (paracellular route), reinforcing the epithelial layer and forming an interconnected network. Thereby the physical barrier takes part in the prevention of antigen penetration across the intestinal epithelium.

2.3.1 Structure of tight junctions

The intercellular tight junctions (TJs) are the most apical component of the intercellular junctional complex, that provides a selective barrier to the movement of substances within the paracellular space.

The TJs, formed in epithelial and endothelial cells as well as in a variety of specialized epithelial cell types, are composed of anastomosing strands of fibrils that completely encircle the apical region of the cell. These fibrils consist of transmembrane proteins that interact with proteins on adjacent cells (Staehelin, 1973). A number of proteins are associated with TJs (fig. 4).

ZO-1, with a molecular weight of 210-225 kDa, is a peripheral membrane associated component, localized in the immediate vicinity of the plasma membrane of TJs and is found ubiquitously within TJs of epithelial and endothelial cells (Stevenson *et al.*, 1986). ZO-1 has been found to interact with the actin cytoskeleton (Tsukamoto and Nigam, 1997). In epithelial cells, ZO-1 forms a heterodimeric complex with the peripheral proteins ZO-2 and ZO-3 (p130) (Gumbiner *et al.*, 1991; Balda and Anderson, 1993). ZO-1 contains an alternatively spliced amino acid domain within its C-terminus, the α-motif, which is correlated with the plasticity of TJs (Balda and Anderson, 1993). Interestingly, *in vivo* the α^+ isoform is expressed in epithelial cells, whereas the α^- isoform is expressed in endothelial cells (Willott *et al.*, 1992). *In vitro* many cell lines express both isoforms (Van Itallie and Anderson, 1999).

Occludin was the first protein found to be associated with TJs (Furuse *et al.*, 1993). Binding to ZO-1 results from interaction with a long cytoplasmic carboxyl-terminal tail of the transmembrane protein (Furuse *et al.*, 1994). Another family of transmembrane proteins contributing to the formation of TJs are claudins. In the intestine the mainly expressed members of this family are claudin 2-5 (Rahner *et al.*, 2001). The structural domains of claudins are similar to occludin. Like occludin, the C-terminus harbors a conserved motif required for the binding to ZO family members (Itoh *et al.*, 1999). Claudins play a role in the regulation of permeability and barrier function (McCarthy *et al.*, 2000).

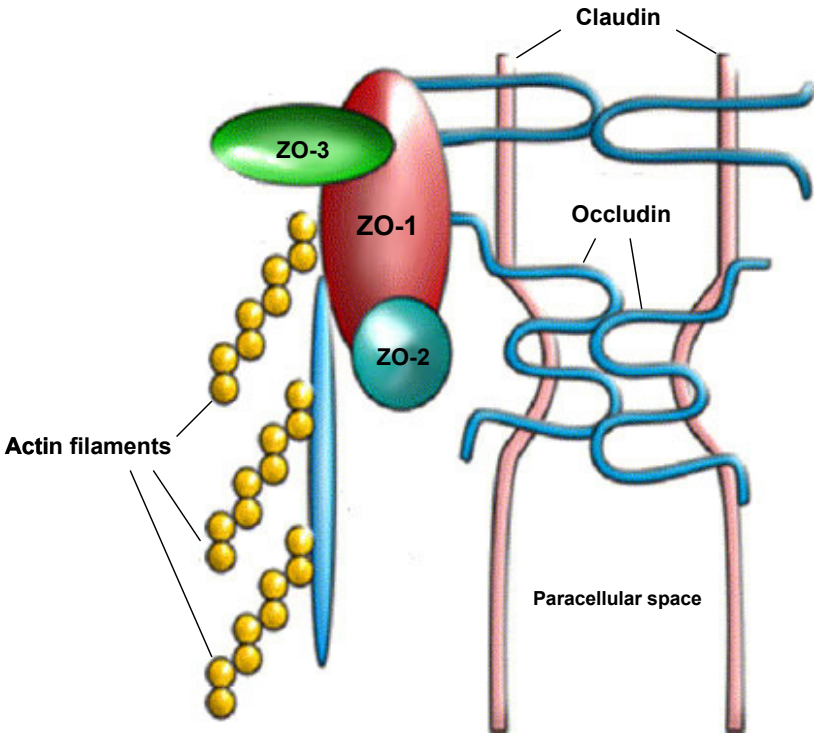

Figure 4. Model for components of the TJ. The transmembrane proteins occludin and claudin are anatomically and functionally connected with the cell cytoskeleton via the junctional complex. This complex comprises a series of proteins, including ZO-1, ZO-2 and ZO-3. (modified from Fasano and Nataro, 2004)

2.3.2 Functions of TJs

ZO-1 not only localizes at the membrane but also to the nucleus under growing cell conditions (Gottardi *et al.*, 1996), suggesting that this molecule is also involved in regulation of gene expression, cell growth and differentation (Balda and Anderson, 1993; Balda and Matter, 2003). Furthermore, TJ associated proteins play a putative role in endocytosis and membrane trafficking (Zahraoui *et al.*, 1994; Kohler *et al.*, 2004). However, the most important function of TJs correspond to permeability properties of epithelial barriers as they restrict diffusion along paracellular space. The formation of the paracellular barrier appears to be directly mediated by occludin, as the increased expression of this TJ associated protein is correlated with enhanced barrier properties in various tissues (Wan *et al.*, 1999; Demaio *et al.*, 2001; Antonetti *et al.*, 2002). Albeit, occludin is not required to maintain the structural integrity of TJs, since the knockout of the occludin gene in mouse embryonic stem cells did not ablate the formation of TJs (Saitou *et al.*, 1998; Saitou *et al.*, 2000). This observation initiated to the search of additional junction proteins and the discovery of claudins interacting with partners on adjacent cells (Tsukita *et al.*, 2001). Functionally this appears to lead to junctions with varying degrees of permeability. A large and growing number of proteins localized to the cytoplasmic face of the TJs are involved in the dynamic regulation of tight junctional properties in response to endogenous

and exogenous stimuli. One of these proteins is ZO-1. The relative abundance of the two ZO-1 isoforms determines whether a given junctions is “tight” or “leaky” (Willott *et al.*, 1992). There is evidence that the abundance and stability of several junctional elements, including occludin and ZO-1, are determined by their phosphorylation status. Dephosphorylated proteins may no longer be retained at the junctional area, and this dissociation therefore leads to a reduction in junction effectiveness (Andreeva *et al.*, 2001). Finally, the TJ barrier is not a static structure, but is subject to minute-to-minute regulation to subserve physiological needs. Besides, a wide array of growth factors, cytokines, drugs, and hormones regulate TJs and permeability (Harhaj and Antonetti, 2004).

2.3.3 Disruption of TJs by microbial pathogens

In contrast to commensal microorganisms, enteric bacterial pathogens subvert, sometimes invade and often cause inflammatory destruction of the intestinal epithelium. Pathogens need to gain access to the epithelial surface to colonize it and to then disrupt this barrier and invade the mucosa, as well as to overcome host-defense mechanisms that are triggered by their aggressive behaviour. The number of ways that pathogens can disrupt and/or exploit TJ proteins expands continously. What is remarkable, however, is the breadth and complexity of strategies developed by enteric bacteria to affect intestinal permeability. Many of these enteric non-invasive pathogens have therefore evolved production of toxins affecting the cytoskeleton, destroying TJ structural elements, and affecting TJ competency by stimulating host signaling events (some mechanisms are summarized in fig. 5).

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) both colonize the epithelium through the close adherence to, and effacement of, the brush border, followed by injection of effectors through a type III secretory system, a molecular syringe, through which the non-invasive microbe delivers bacterial proteins directly into the host cells. EHEC also produces cytokines known as Shiga toxins. Both pathogens affect cytoskeletal contraction and thereby enhance TJ permeability (Hecht *et al.*, 1996; Turner *et al.*, 1997; Yuhan *et al.*, 1997; Turner *et al.*, 2000; Berglund *et al.*, 2001). Additionally, Philpott et al. have shown that EHEC infection of IECs decreases transepithelial resistance and induces altered barrier function through disruption of ZO-1 protein (Philpott *et al.*, 1998).

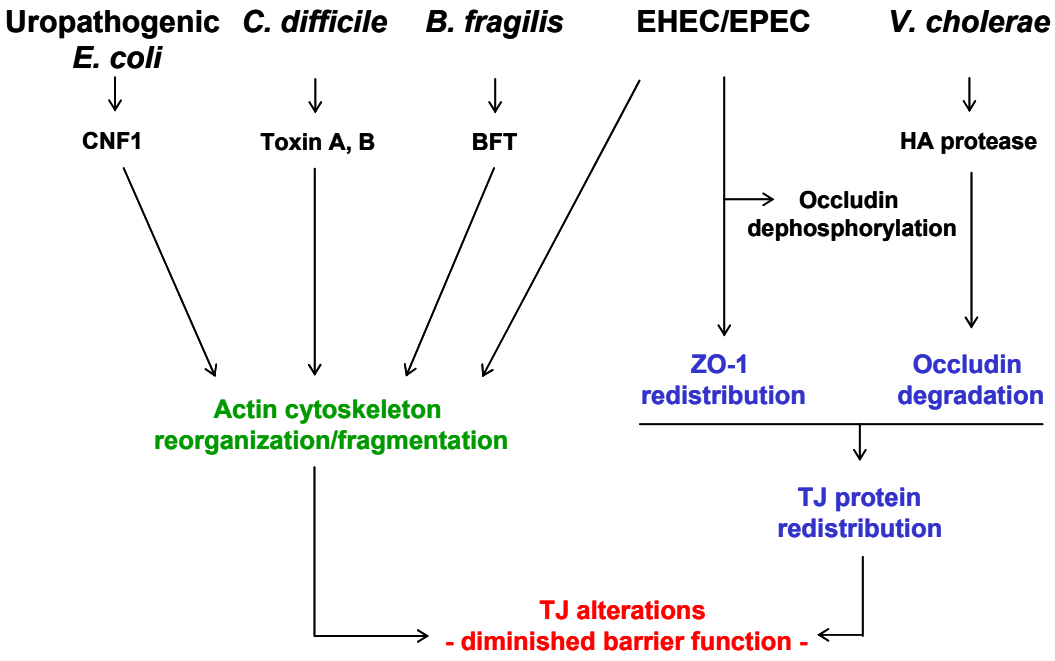

Figure 5. Epithelial TJs can be altered by various pathogens, as well as by their elaborated toxins.
These effects may result from direct modification of TJ proteins such as occludin, claudin, and ZO-1, or by affecting the cytoskeletion complex. BFT, *Bacteroides fragilis toxin*; CNF, cytotoxic necrotizing factor; *E. coli*, *Escherichia coli*; *EPEC*, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; HA, hemagglutinin. (adapted from Berkes *et al.*, 2003)

Infection of IECs with EPEC causes disassembly of the TJ complex with a progressive decrease in the protein-protein interactions within the TJ. This is accompanied by a dramatic redistribution of apically localized TJ proteins to a disorganized array of TJ strands along the lateral membrane (Muza-Moons *et al.*, 2004).

Some pathogenic microorganisms like *Clostridium difficile*, the pathogen responsible for antibiotic-associated pseudomembranous colitis (Dillon *et al.*, 1995; Borriello, 1998; Nusrat *et al.*, 2001) and uropathogenic *Escherichia coli* strains (Boquet, 2001) colonize the epithelial surface and cause inflammation through the production of cytotoxins. These cytotoxins affect the actin cytoskeleton, which results in alterations of the TJs. Same results are effected - directly or indirectly - by bacteria derived proteases like the enterotoxin of *Bacteroides fragilis* (BFT) (Wu *et al.*, 1998) and the hemagglutinin protease (HA/P) of *Vibrio cholerae* (Wu *et al.*, 2000).

2.3.4 The role of the intestinal barrier in diseases

The inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract. The pathogenesis of IBDs remains unknown, although there is convincing evidence to implicate genetic, immunological, and environmental factors in the initiation of the autoimmune process (Danese *et al.*, 2004).

Several lines of evidence suggest that increased intestinal permeability has a central role in the pathogenesis of IBDs. For example, between 10 and 20% presymptomatic CD patients have been shown to exhibit increased gut permeability (Meddings, 1997; Hollander, 1999). Alteration of TJ structure in UC for instance results in impaired barrier function (Schmitz *et al.*, 1999). Localization studies in mucosal biopsies from IBD patients have revealed disappearance of key TJ proteins from intercellular junctions (Barmeyer *et al.*, 2004; Ivanov *et al.*, 2004).

In vitro experiments examining the effects of inflammatory cytokines on IECs revealed that the production of cytokines, including interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), perpetuates the increased intestinal permeability by recognizing ZO-1, occludin, claudin 1 and claudin 4 (Ma *et al.*, 2005; Wang *et al.*, 2005). Interestingly, disruption of epithelial barrier function is associated with internalization of transmembrane TJ proteins JAM1, occludin and claudins 1 and 4 (Ivanov *et al.*, 2004).

Celiac disease, an autoimmune digestive disorder, is characterized by intestinal epithelial damage caused by exposure to gluten and related proteins found for instance in wheat, leading to a dysfunction of nutrients absorbance. Early in the development of celiac disease TJs are opened (Madara and Trier, 1980; Schulzke *et al.*, 1998), most likely secondary to zonulin up-regulation (Fasano *et al.*, 2000), induced by exposure to gliadin (Clemente *et al.*, 2003), and severe intestinal damage ensues (Schulzke *et al.*, 1998).

3. Gastrointestinal immune system

The intestinal immune system is the largest and most complex part of the immune system. Although strong immune responses are required to protect the mucosal surface against pathogens, equivalent responses against non-pathogenic materials would be wasteful, and hypersensitivity responses against dietary antigens or commensal bacteria can lead to inflammatory disorders. These responses have to be prevented by a complex interplay of regulatory mechanisms that ensure the maintenance of homeostasis in the gut.

3.1 GALT - the inductive sites for mucosal immune responses

The major inductive site for mucosal immune responses of the gastrointestinal tract is the gut-associated lymphoid tissue (GALT) that can be divided into effector sites consisting of lamina propria lymphocytes, scattered throughout the epithelium and the intraepithelial lymphocytes (IEL) that are embedded in the epithelial cell layer, as well as inductive sites responsible for the induction phase of the immune response (fig. 6).

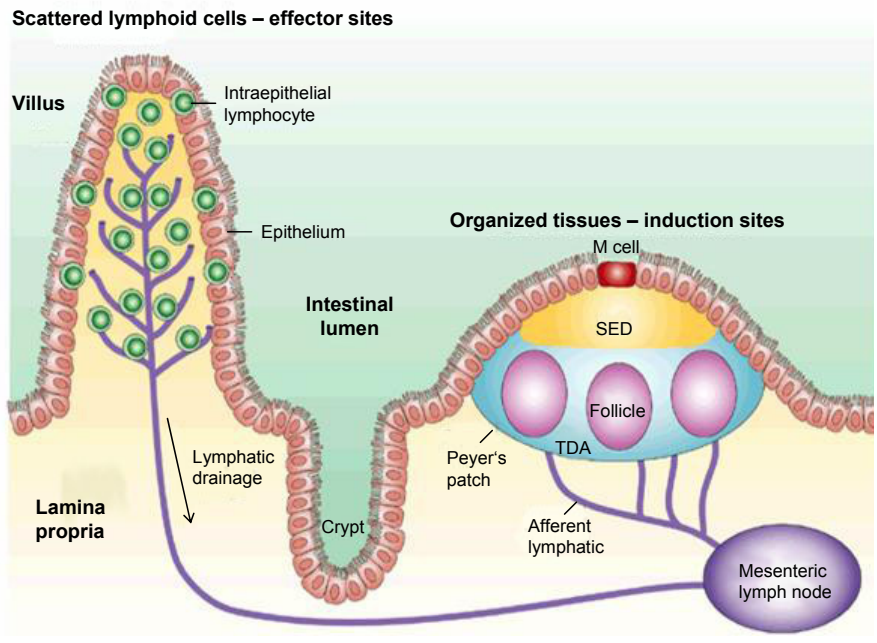

Figure 6. Schematic presentation of the lymphoid elements of the intestinal immune system. The organized tissues of the Peyer's patches and mesenteric lymph nodes are involved in the induction of immunity and tolerance, whereas the effector sites are scattered throughout the lamina propria and epithelium of the mucosa. SED, subepithelial dome; TDA, thymus-dependent area. (adapted from Mowat, 2003)

This inductive site of the GALT is separated from the intestinal lumen by a single layer of epithelial cells, known as follicle-associated epithelium (FAE), and a more diffuse area immediately below the epithelium, the subepithelial dome (SED). The most notable feature of the FAE is the presence of microfold (M) cells. These are

specialized epithelial cells that lack surface microvilli and the normal thick mucus layer. Their primary function is to act as specific ports for antigens, facilitating the uptake of luminal antigens and their subsequent delivery to the initiation compartment of the organized lymphoid tissues, the Peyer's patches and mesenteric lymph nodes (MLNs) (fig. 6). Following induction of the GALT and stimulation in the initiation compartment, numerous antigen-experienced T cells migrate to the lamina propria, the main effector compartment, and on the other side to the intestinal epithelium, where they reside interspersed among the luminal epithelial cells.

3.2 IECs – the effector sites for mucosal immune responses

Immediately after birth epithelial surfaces are coming into contact with vast numbers of microorganisms. These surfaces therefore evolved a number of protective mechanisms to resist invasion of microorganisms. Disruption of the physical barrier, formed by IECs and TJs, enables the transit of molecules, bacteria and nutrients across the epithelium, resulting in the induction of immune responses. Effective immune responses to antigenic proteins require the help of T lymphocytes. Stimulation of T cells in turn depends on exogenous antigens being presented by antigen-presenting cells (APCs).

3.1.1 IECs acting as non-professional APCs

IECs take an active part in the induction of adaptive immune surveillance at the mucosal surface via collaboration with professional APCs like dendritic cells, macrophages, B cells and lymphoid cells, mainly in the FAE. But there is evidence, that IECs themselves act as non-professional APCs (Mayer, 1998; Blumberg *et al.*, 1999; Hershberg and Mayer, 2000). First, IECs are located at the interface of the largest antigenic load in the body and the largest population of lymphocytes - thus separating them from one another. Since the 1980s it was noted, that IECs similar to professional APCs constitutively express several surface molecules like major histocompatibility complex (MHC) class I and II molecules that are relevant to antigen processing and presentation to T cells. In addition, these observations suggest IECs to influence antigen presentation to lamina propria lymphocytes even without direct contact.

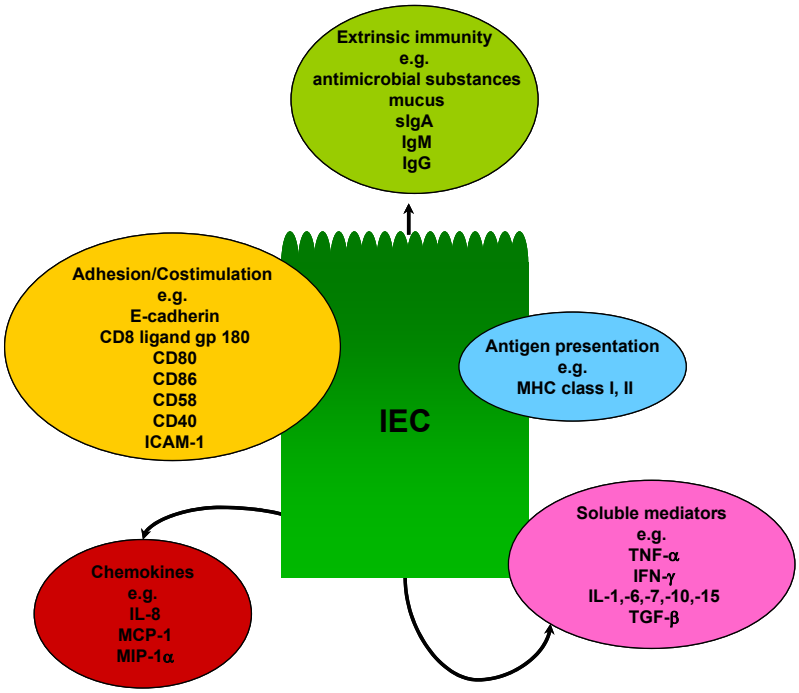

Figure 7. Model depicting the various means by which IECs interact with cells of the surrounding mucosal immune system.
IFN, interferon; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibility complex; MIP, macrophage inhibitory protein; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; TGF, transforming growth factor. (adapted from Pitman and Blumberg, 2000)

In contrast to professional bone-marrow derived APCs, IECs are not equipped with the full setting of antigen-presenting and costimulatory molecules. Under normal conditions IECs do not express the costimulatory molecules CD80, CD86 or CD40, but do express CD58 (Framson *et al.*, 1999) and a CD8 ligand gp 180 (Yio and Mayer, 1997). Whereas in IBDs, IECs are induced to express CD86 (Nakazawa *et al.*, 1999), and infected IECs do express intercellular adhesion molecule-1 (ICAM)-1) (Huang, 1996) (fig. 7).

3.1.2 Chemokine and cytokine secretion by IECs under non-inflammatory conditions

IECs cells also take place in the intestinal innate immune responses by secretion of mediators – cytokines and chemokines (fig. 7).

The group of cytokines secreted by IECs that are involved in regulating lymphocyte development and growth include among others TGF-β (Ciacci *et al.*, 1993), IL-6 (Panja *et al.*, 1995), IL-7 (Madrigal-Estebas *et al.*, 1997) and IL-15 (Reinecker *et al.*, 1996). In addition, when sensitized with leukocytes, IECs also express TNF–α (Haller *et al.*, 2000), thought to be only expressed after challenge with enteropathogens (Jung *et al.*, 1995). An increased TNF-α release could also be observed by IECs in response to non-pathogenic coliform bacteria (Borruel *et al.*, 2003). Although early studies suggested that the production of chemokines by IECs may require invasion of the epithelial cells by bacterial pathogens, it has become

clear that this is not necessarily the case (Crabtree and Farmery, 1995). IECs mainly secrete CXC chemokines, monokine induced by IFN-γ (MIG), IFN-γ-inducible protein 10 (IP-10), and chemokine (C-X-C motif) ligand 11 (I-TAC) (Dwinell *et al.*, 2001; Shibahara *et al.*, 2001). The CC chemokines macrophage inflammatory protein-3α (MIP-3α) (Neutra *et al.*, 2001) and chemokine (C-C motif) ligand 28 (CCL28) (Ogawa *et al.*, 2004) are also expressed and produced by IECs. Signals mediated by MIP-3α receptor CCR6, expressed by IECs, result in increased IEC migration and proliferation, suggesting an important role in intestinal homeostasis and inflammation (Brand *et al.*, 2005). Further expressed CC and CXC chemokine receptors are CCR1-8 and CXCR4 (Dwinell *et al.*, 1999). Another constitutively expressed CC chemokine is the monocyte chemoattractant protein 1 (MCP-1) (Reinecker *et al.*, 1995). MCP-1 is suggested to play a major part in IBDs as it is noticeably increased under these intestinal inflammatory conditions (Reinecker *et al.*, 1995). This molecule is known to activate macrophages and increase the migration of monocytes into tissue during inflammation. It has been reported, that infection of IECs with invasive bacterial strains (*Salmonella dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *Listeria monocytogenes*, enteroinvasive *Escherichia coli*) resulted in the coordinate expression and up-regulation of MCP-1 (Jung *et al.*, 1995). Similar results were obtained for *Campylobacter jejuni*, a common cause of diarrhea (Hu and Hickey, 2005). The increased MCP-1 production in response to stimulation of IECs with IL-1β and TNF-α is down-regulated by the immunoregulatory cytokines IL-4, IL-10 and IL-13, described to exert antiinflammatory properties on various cell types (Kucharzik *et al.*, 1998).

The expression and secretion of several cyto – and chemokines by IECs emphazise their participation in immune regulation in the mucosa.

4. Intestinal microflora

Many bacterial species have evolved and adapted to live and grow in the human intestine. The intestinal habitat of an individual contains 300-500 different species of bacteria (Simon and Gorbach, 1984), and the number of microbial cells within the gut lumen is about 10 times larger than the number of eukaryotic cells in the human body (Bengmark, 1998). The indigenous microbiota (commensal microflora) consists of those microorganisms, which are present on body mucosal surfaces and are exposed to the external environment. The microflora has an important role in human nutrition and health, promoting nutrient supply, preventing pathogen colonization and shaping and maintaining normal mucosal immunity (Xu *et al.*, 2003). Evidence that host nutrition is supplemented by the metabolic capabilities of the resident microflora is derived from studies on mono-associated and conventionalized mice, which, unlike germ-free animals, are able to capture and store energy, extracted by bacterial degradation of undigested dietary substrates (Backhed *et al.*, 2004).
Commensal bacteria coevolved with their hosts, however, under specific conditions they are able to overcome protective host properties and exert pathologic effects.

4.1 Composition of the commensal microflora

Colonization of the gastrointestinal tract of newborn infants starts immediately after birth and occurs within a few days. Initially, the type of delivery and the type of diet might affect the colonization pattern (Gronlund *et al.*, 1999; Harmsen *et al.*, 2000).
The most abundant microflora is present in the distal parts of the gut; more than 90% of the intestinal bacteria are Gram-negative anaerobes. The genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* are predominant in humans (Stephen and Cummings, 1980), whereas aerobes (facultative anaerobes) such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus* are among the subdominant genera. The species vary greatly between individuals (Moore and Moore, 1995). The gastric acidity of the stomach creates an environment that is inimical to microbial survival (Hentges, 1993) and is therefore in general free of a microflora. However, one bacterial species, *Helicobacter pylori*, has the capacity to overcome the aciditiy and to stably colonize gastric mucosal epithelium (Lee and O'Rourke, 1993). The two major parts of the intestine, the small intestine and the colon, differ profoundly in their bacterial loads (tab. 1). In contrast to low bacterial numbers in small intestine (10^{3-9}

bacteria per gram of gut content), the highest numbers of bacteria displaying enormous diversity are found in the colon – the bacterial populations can exceed 10^{14} per gram of feces (Hao and Lee, 2004). Indeed, the mucosal surface and the lumen of the large intestine harbor 99,9% of the human indigenous microbiota.

Table 1. Most frequent bacterial species of the human microbiota.

	Density	Bacterial species
Stomach	0-10^5/g	Flora of the mouth
		Lactobacilli
		Gram-positive cocci
Duodenum	10^3/g	Equal to stomach
Jejunum	10^5/g	Lactobacilli
		Streptococci
		A few anaerobic bacteria
Ileum	10^8 – 10^9/g	Gram-positive cocci
		Enterobacteriaceae
		Bacteroides spp.
		Anaerobic bacteria
Colon	10^{11} – 10^{14}/g	*Bacteroides* spp.
		Clostridium spp.
		Bifidobacteria
		Eubacteria
		Lactobacilli
		Enterobacteriaceae
		Fusobacteria
		Streptococci
		Enterococci
		Staphylococci

4.2 Functions of the microflora

The housing of animals under germ-free conditions has generated important information about the effect of the microbial composition of the gut on host physiology and pathology (Falk *et al.*, 1998). For instance, compared to conventionally reared counterparts, germ-free rodents showed a greatly enlarged cecum, thinner intestinal walls, reduced overall cell mass and less IgA plasma cells in the gut (Crabbe *et al.*, 1968; Gordon and Pesti, 1971; Thompson and Trexler, 1971). Evidence obtained from such studies suggests that the intestinal microflora has important and specific metabolic, trophic and protective functions (Roberfroid *et al.*, 1995). A major

metabolic function of the colonic microflora is the fermentation of non-digestible dietary residues: salvage of energy as short-chain fatty acids, production of vitamin K, absorption of ions (Roberfroid *et al.*, 1995). Possibly, the most important role of short-chain fatty acids on colonic physiology is their trophic effect on the intestinal epithelium. The rate of production of crypt cells is reduced in colon of rats bred germ-free, and their crypts contain fewer cells than those of animals colonized by conventional flora, indicating that intraluminal bacteria affect cell proliferation in the colon. It has been shown, that fermentable fibre leads to an increase of crypt numbers in the colon in the presence of microflora (McCullogh *et al.*, 1998). Differentiation of epithelial cells is greatly affected by interaction with resident microorganisms. Studies regarding colonization of germ-free mice with gut commensals revealed that these microorganisms are able to modulate expression of host genes that participate in diverse and fundamental physiological functions (Hooper *et al.*, 2001). Furthermore, the use of germ-free animals revealed the impact of the indigenous microflora on the immune system. Animals bred in a germ-free environment have low densities of lymphoid cells in the gut mucosa, specialized follicle structures are small and circulating concentrations of immunoglobulins in the blood are low (Falk *et al.*, 1998; Butler *et al.*, 2000; Tannock, 2001). Microbial colonization of the gastrointestinal tract affects the composition of GALT. Immediately after exposure to luminal microbes, the number of intraepithelial lymphocytes expands greatly (Umesaki *et al.*, 1993; Helgeland *et al.*, 1996), germinal centres with immunoglobulin producing cells arise rapidly in follicles and the lamina propria (Cebra *et al.*, 1998), and concentrations of immunoglobulin increase substantially in serum (Butler *et al.*, 2000). Thus, controlled inflammation is the result of bacterial colonization.

4.3 The indigenous microflora and the intestinal immune system

Through their metabolic activities, the intestinal flora has a significant impact on the well-being of the host. Not only the metabolism of the resident microflora probably influences the health but also the continuous stimulation of the GALT by a variety of antigenic components which are believed to mediate tolerogenic as well as immunogenic stimuli.

The normal intestinal epithelium must maintain hyporesponsiveness toward the commensal bacteria that are constantly present in the lumen (Xavier and Podolsky, 2000) (fig. 8).

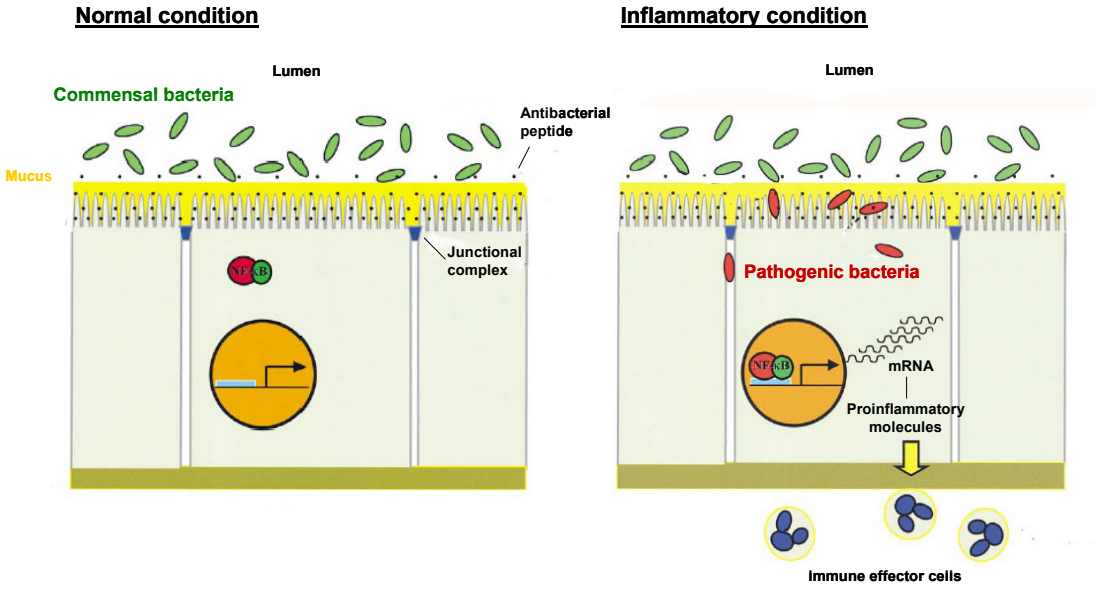

Figure 8. Schematic comparison of the intestinal epithelium under normal and inflammatory conditions.
Under normal conditions the intestinal epithelium can deploy a number of non-specific defenses to maintain coexistence with the normal flora, including selectively permeable junctional complexes and secretion of mucus and anti-bacterial peptides. Under inflammatory conditions intestinal epithelium can orchestrate an inflammatory response. Enteropathogenic bacteria are able to disrupt junctional complexes or physically invade into inter- or intracellular compartments. These events result in activation of signaling cascades and induction of proinflammatory gene expression. (adapted from Neish, 2002)

It has been demonstrated that commensal bacteria and their non-pathogenic constituents (commensal-associated molecular pattern – CAMP) may directly "switch off" downstream signal transduction pathways in IECs via NF-κB to maintain mucosal homeostasis and prevent excessive inflammatory responses (Neish *et al.*, 2000). Pathogenic bacteria are also able to directly deposit their toxic and proinflammatory constituents including their cell-wall-specific signatures (pathogen-associated molecular pattern – PAMP) such as lipopolysaccharide (LPS), a glycolipid derived from the outermost membrane of pathogenic Gram-negative bacteria, at the intestinal epithelial apical surface.

To coexist with the milieu of the gut, the host has evolved survival and active defense features: The intestine's primary protection from the broad microbial ecosystem is likely the highly selective physical barrier formed by the epithelium with the intracellular TJs. In addition to this anatomic barrier, the intestinal epithelium

functions as a highly efficient immunologic barrier exhibiting a combination of adaptive immune features and maintaining luminal homeostasis through controlled inflammatory responses *in vivo* (Xavier and Podolsky, 2000).

4.3.1 Recognition of commensal bacteria by IECs – the role of TLR

The innate immune system of mucosal surfaces comprises a large number of cell populations (e.g. IECs, dendritic cells, M cells), producing cytokines essential for inflammatory reactions as well as factors critical for the subsequent initiation of specific immunity. Innate immunity recognition is mediated by a structurally diverse set of receptors, so-called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). A recently recognized family of PRRs are the nucleotide-binding oligomerization domain (NOD) proteins, which are expressed intracellularly and recognize bacterial peptidoglycans. NOD1 recognizes muramyl tripeptides from Gram-negative bacteria, whereas NOD2 recognizes muramyl dipeptide, that is common to the peptidoglycans of all bacterial species, regardless of their Gram-staining characteristics (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a and 2003b). Among these, the family of so-called membrane-bound Toll-like receptors (TLRs), which are found to be expressed in many cell types throughout the whole gastrointestinal tract *in vivo* and *in vitro*, play an important role.

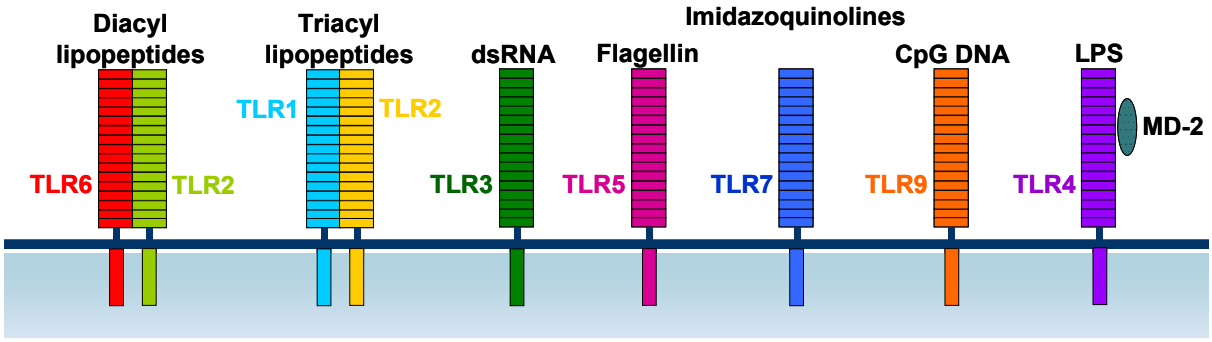

Figure 9. TLRs and their ligands.
TLR2 is essential in the recognition of microbial lipopeptides and lipoproteins. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in cytosine-guanine (CpG) DNA recognition, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 recognizes flagellin. (modified from Takeda and Akira, 2004)

Until now 11 different TLRs have been identified, whereas TLR11 is only present in mice intestine (Abreu *et al.*, 2005; Lauw *et al.*, 2005). TLRs function as sensors of microbial infection and are critical for the initiation of inflammatory and immune defense responses.

Mammalian TLRs enable IECs to participate in innate immunity to microorganisms in at least four ways: recognition of CAMPs and PAMPs (fig. 9), expression at the interface with the environment of the gastrointestinal lumen, induction of secretion of pro- and antiinflammatory cytokines and chemokines that link to the adaptive immune system and to the induction of antimicrobial effector pathways.

PAMP activation of most TLR induces intracellular signaling cascades. The TLR signaling pathways are distinguished in MyD88-dependent and MyD88-independent pathways (fig. 10).

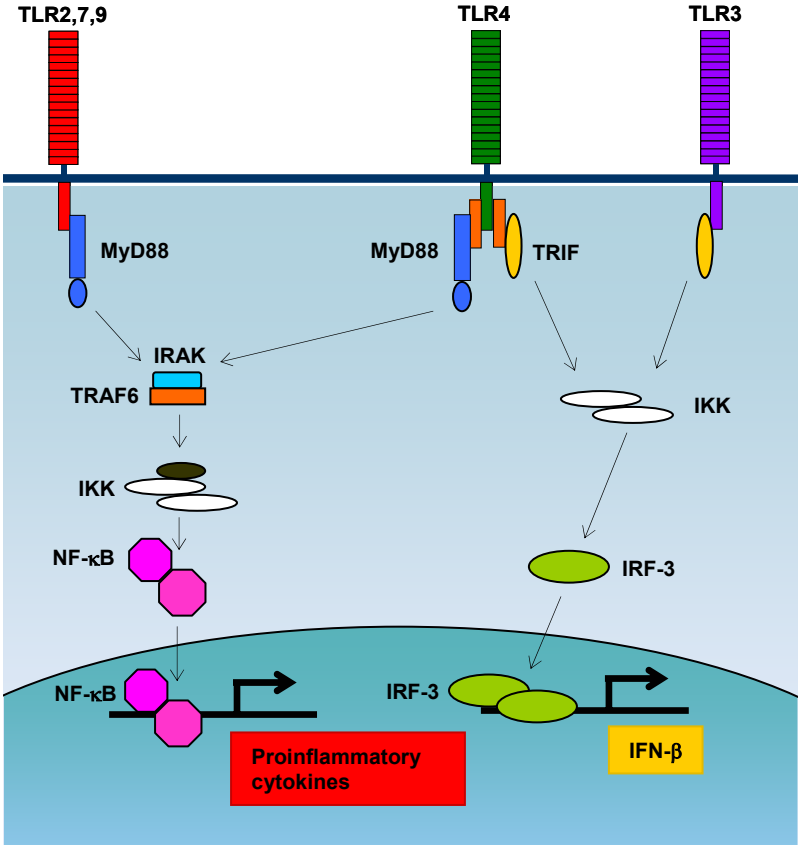

Figure 10. Simplified schematic presentation of the MyD88-dependent and -independent pathways. Upon stimulation, the cytoplasmic Toll/IL-1 receptor (TIR) domain-containing adaptor MyD88 recruits the serine/threonine kinase IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of both molecules. IRAK becomes activated and then associates with TNF-receptor associated factor 6 (TRAF6), leading to NF-κB activation. For TLR3- and TLR4-mediated MyD88-independent pathways the essential molecule is the TIR domain containing adaptor TRIF (TIR domain containing adaptor inducing IFN-β) leading to activation of the transcription factor interferon regulatory factor 3 (IRF-3) and subsequent induction of IFN-β. (modified from Takeda and Akira, 2005)

MyD88 is essential for the production of proinflammatory cytokines mediated by all TLR family members. In accordance with the lack of production of inflammatory cytokines, no NF-κB activation in response to TLR2, TLR7 and TLR9 ligands was observed in MyD88 knockout mice. However, TLR4 ligand-induced activation of NF-κB was observed, although it was delayed compared with that in wild-type mice (Kawai *et al.*, 1999). These findings indicate the presence of a MyD88-independent component of the signaling pathway downstream of TLR4 (Kawai *et al.*, 2001; Doyle *et al.*, 2002; Hoshino *et al.*, 2002; Toshchakov *et al.*, 2002). In addition to TLR4

utilizing the MyD88-independent pathway, TLR3 signaling also leads to the activation of interferon regulatory factor 3 (IRF-3) and subsequent induction of type I interferons (IFNs) like IFN-β (Takeda and Akira, 2005).

Importantly, the bacterial ligands recognized by TLRs are not unique to pathogens, but rather are shared by entire classes of bacteria, and are therefore produced by commensals as well. So the question arises, why mucosal surfaces colonized by bacteria are not in permanent inflammatory state. Three prinicipal factors are expected to account for tolerance to commensal microorganisms: properties of the bacteria themselves (e.g. impaired ability to escape trapping in mucus and to adhere and invade the epithelial barrier, low endotoxicity), characteristics of the epithelial surface (e.g. defective sensing of molecular PAMPs as a result of reduced expression for example of TLRs) and properties of the immune cells that are present in the lamina propria (e.g. tolerogenic APCs and regulatory T cells that produce antiinflammatory cytokines in response to commensal bacteria) (Sansonetti, 2004). Commensal bacteria are known to have the ability to express lower amounts of PAMPs than pathogenic bacteria and/or produce molecules with immunosuppressive effects. It is thought, however, that the absence of TLRs from the apical surfaces of epithelial cells, either owing to their intrinsic polarization to basolateral surfaces or as a result of ligand-induced down-regulation, might contribute to the hyporesponsive tone of the gut towards its diverse microflora (Kelly *et al.*, 2005). Additionally, in contrast to commensal microorganisms, pathogenic bacteria are equipped with virulence factors that allow them to pass through epithelial barriers where they can be detected by TLRs expressed on macrophages and dendritic cells (Gewirtz *et al.*, 2001; Sansonetti, 2002). Furthermore, commensals in contrast to pathogens down-regulate proinflammatory responses by several mechanisms (e.g. inhibition of I-κB ubiquitination) (Neish *et al.*, 2000; Kelly *et al.*, 2004). The importance of stimulation of the innate immune system by commensal bacteria was demonstrated by the recent finding that recognition of commensal bacterial products by TLRs results in resistance to epithelial injury and maintenance of epithelial homeostasis. Thus, TLRs have at least two distinct functions depending on the recognition of pathogens and commensals: protection from infection and control of tissue homeostasis (Rakoff-Nahoum *et al.*, 2004).

4.4 The microflora in intestinal disease

Many clinically relevant diseases have been linked with dysfunctional immune responses directed against the commensal microflora. Such aberrant immune responses, in conjunction with genetic predisposition, contribute to the pathogenesis of IBDs (Oostenbrug *et al.*, 2003). Furthermore, the hygiene hypothesis, which attempts to explain the rising incidence of atopic, autoimmune and inflammatory diseases among the human population, postulates that the innate immune response to commensal bacteria influences the adaptive response to both food and environmental antigens (Sartor, 2004). Hence, an imbalance between aggressive and protective bacterial species, or loss of gut bacteria that promotes tolerance and regulatory T cell polarization, could lead to excessive T helper (T_H) 1 and T_H2 responses, thus promoting inflammatory or autoimmune diseases or allergic diseases, respectively. Interestingly, experimental colitis and IBDs occur in regions of highest bacterial stimulation; the ileum and the cecum.

In patients with CD, an idiopathic chronic disorder characterized by patchy transmural inflammation of the gastrointestinal tract (Crohn, 1967), the balance between responsiveness to pathogens and deliberate non-responsiveness to commensal microflora is disturbed (Neurath *et al.*, 2002). Although it can affect any part of the gastrointestinal tract, it most commenly involves the ileum and colon. The intestinal T lymphocytes in these patients are hyperreactive against bacterial antigens and it was suggested that local tolerance mechanisms are abrogated in such patients (Pirzer *et al.*, 1991). Moreover, patients with IBDs have higher amounts of bacteria attached to their epithelial surfaces than do healthy people and additionally some of the bacteria, especially *Bacteroides* spp., were identified within the epithelial layer, and in some instances, intracellularly (Swidsinski *et al.*, 2002). Some patients with CD (17-25%) have mutations in the NOD2/CARD15 gene, which regulates host responses to bacteria (Hampe *et al.*, 2001; Russell *et al.*, 2005). New data on NOD2/CARD15 function and NF-κB activation indicate that an inflammatory reaction of the intestinal mucosa as a response of the innate immune system may be necessary for the maintenance of gut homeostasis. CD may therefore be seen as a defective immune response and no longer only as hyperresponsiveness of the mucosal immune system (Rogler, 2004).

In the last decade, various experimental mouse models with induced mutations of host genes have been developed, resulting in an impaired homeostasis with the

intestinal microbiota leading to IBDs - like CD and UC. Most models used conventionally reared animals that exhibit some sort of immune dysregulation. Common to these models is that induction of colitis does not develop when these mice were raised under germ-free conditions, indicating that pathological consequences depend on stimulation of innate or adaptive immunity by commensal antigens (Wirtz and Neurath, 2000).

5. Probiotics

There is strong evidence of a role for the indigenous flora in driving inflammatory immune responses in IBDs in genetically predisposed individuals (Guarner *et al.*, 2002). Researchers have tried to identify a specific pathogen as the cause of these chronic intestinal inflammatory disorders but the possibility that one or more bacterial agents are responsible cannot be ruled out. Considering the implications of a pathogen in IBDs, it was hypothesized that modulation of an imbalanced microflora in these patients by introducing high titres of "protective" bacteria might overwhelm the "aggressive" strains or might lead to compensation of bacterial loss. On this basis, probiotic treatment was proposed as a therapeutic approach (Campieri and Gionchetti, 1999).

5.1 History of probiotics

The term probiotic is a relatively new word meaning "for life" and it is currently used to name bacteria associated with beneficial effects for humans and animals. The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian born Nobel Prize recipient working at the Pasteur Institute at the beginning of the last century, who suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907). At this time Henry Tissier, a French paediatrician, observed that children with diarrhea had low numbers of bacteria in their stools characterized by a peculiar, Y shaped morphology. These "bifid" bacteria were, on the contrary, abundant in healthy children (Tissier, 1906). He suggested that these bacteria could be administered to patients with diarrhea to help restore a healthy gut flora.

The works of Metchnikoff and Tissier were the first to make scientific suggestions about the probiotic use of bacteria, even if the word "probiotic" was not coined until 1960, to name substances produced by microorganisms which promoted the growth of other microorganisms (Lilly and Stillwell, 1965). In order to point out their microbial nature, probiotics were redefined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). In 1992 Havenaar and Huis in't Veld defined the term probiotic as "A viable mono or mixed culture of bacteria which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora" (Havenaar and Huis in't Veld,

1992). The latest and most appropriate definition describes probiotics as “Live microorganisms which, when consumed in adequate amounts, confer a health effect on the host” (Guarner and Schaafsma, 1998; Araya *et al.*, 2001). In the last 20 years, research in this area has gained increasing interest and progressed considerably.

5.2 Probiotic microorganisms

For the use of probiotic microorganisms in foods, they should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut. Consequently, these microorganisms have to be resistant to gastric juices and be able to grow in the presence of bile under anaerobic conditions. These demands are mainly - but not exclusively - met by members of the Gram-positive bacteria of the genera *Lactobacillus* and *Bifidobacterium*, members of the normal healthy human intestinal microflora and used extensively as probiotics (tab. 2) (Holzapfel *et al.*, 2001).

Table 2. Examples of microorganisms that are considered to be probiotics.

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Others**
L. acidophilus	*B. bifidum*	*Escherichia coli* Nissle 1917
L. casei	*B. breve*	*Saccharomyces boulardii*
L. crispatus	*B. infantis*	*Streptococcus thermophilus*[1]
L. delbrueckii subsp. *bulgaricus*[1]	*B. longum*	*Enterococcus faecium*[2]
L. fermentum	*B. lactis*	
L. grasseri	*B. adolescentis*	
L. johnsonii		
L. paracasei		
L. plantarum		
L. reuteri		
L. rhamnosus		

[1] There is still debate about the probiotic acitivity.
[2] Safety concerns remain because of potential pathogenicity and vancomycin resistance.
(adapted from Senok *et al.*, 2005)

But also microorganisms of other genera, such as *Escherichia coli* (*E.coli*) Nissle 1917 and the yeast *Saccharomyces boulardii* (*S. boulardii*) are marketed as probiotics (Holzapfel *et al.*, 2001). Prior to undertaking *in vivo* trials, proper *in vitro* studies have to establish the potential health benefits of probiotics. For subsequent *in vivo* testing, randomized, double-blind, placebo controlled human studies should confirm the efficacy of a probiotic (Araya *et al.*, 2001).

5.3 The relevance of probiotics as therapeutic alternatives in human diseases

Fermented dairy products enriched with probiotic bacteria have developed into one of the most successful categories of functional foods (Saxelin *et al.*, 2005). Another increasing administration area for probiotics is the use as therapeutic alternatives in maintaining human health. This development of novel therapies is strengthened by growing problems regarding antibiotic resistance and secondary effects of conventional therapeutics.

Investigations of the past years have convincingly revealed the beneficial impact of probiotics on diverse disorders associated with the gastrointestinal tract. The positive effect of probiotics in reducing the incidence or duration of certain diarrheal illnesses is the most substantiated health claim to date - the emphasis here lies in the use of *Lactobacillus rhamnosus*, *Bifidobacterium lactis* and *Lactobacillus reuteri* in the prevention and treatment of rotavirus diarrhea in children (Phuapradit *et al.*, 1999; Guandalini *et al.*, 2000; Rosenfeldt *et al.*, 2002; Sullivan and Nord, 2005). Further data also suggest beneficial effects of probiotics in other conditions like gastric infections by *Helicobacter pylori* (Cruchet *et al.*, 2003; Pantoflickova *et al.*, 2003), allergy (Kalliomaki *et al.*, 2003; Kalliomaki and Isolauri, 2004) and genitourinary tract infections (Kontiokari *et al.*, 2001; Reid, 2001; St Amant, 2002).

The discovery of the role of enteric microflora as an outstanding factor in the pathogenesis of IBDs has greatly stimulated the interest in probiotics and the scientific evaluation of their use as means to reconstitute microbial and immunological homeostasis. Several clinical trials suggest that selected probiotic species, alone or in combination, can prevent recurrent intestinal inflammation and possibly treat active IBDs. An overview of trials of probiotic agents in IBDs is given in table 3. There is a limited number of randomized, double-blind controlled studies that have shown superiority or equal effects of probiotics compared with placebo or standard medication in the maintenance of remission of CD or UC (Schultz *et al.*, 2003; Sartor, 2004).

Effectively tested probiotic strains in IBDs are *Lactobacillus rhamnosus* GG, *E. coli* Nissle 1917 (Mutaflor®), the bacterial mixture VSL#3 (consisting of four Lactobacilli strains, three Bifidobacteria strains and a single *Streptococcus* strain) and the yeast *S. boulardii* (Perenterol®).

Table 3. An overview of trials of probiotic agents in IBDs.

Author	Probiotic	Result
Ulcerative colitis		
McCann *et al.*, 1994	*E. coli* Nissle 1917, *Lactobacillus acidophilus* DDS-1, *Bifidobacterium bifidum* Malyoth	Maintenance of remission
Kruis *et al.*, 1997	*E. coli* Nissle 1917	Maintenance of remission as effective as mesalazine
Rembacken *et al.*, 1999	*E. coli* Nissle 1917	Maintenance of remission as effective as mesalazine
Venturi *et al.*, 1999	VSL#3	Maintenance of remission
Kruis *et al.*, 2001	*E. coli* Nissle 1917	Maintenance of remission as effective as mesalazine
Ishikawa *et al.*, 2003	Bifidobacteria-fermented milk	Maintenance of remission
Guslandi *et al.*, 2003	*S. boulardii* in combination with mesalazine	Maintenance of remission more effective than mesalazine alone
Fedorak *et al.*, 2003	VSL#3	Maintenance of remission
Tursi *et al.*, 2004	VSL#3 in combination with balsalazide	Maintenance of remission more effective than balsalazide alone
Kruis *et al.*, 2004	*E. coli* Nissle 1917	Maintenance of remission as effective as mesalazine
Cui *et al.*, 2004	Bifidobacteria after sulfasalazine and glucocorticoid treatment	Reduction of relapse, decrease of inflammatory activity
Bibiloni *et al.*, 2005	VSL#3	Maintenance of remission
Crohn's disease		
McCann *et al.*, 1994	*E. coli* Nissle 1917, *Lactobacillus acidophilus* DDS-1, *Bifidobacterium bifidum* Malyoth	Maintenance of remission
Malchow, 1997	*E. coli* Nissle 1917 after prednisolone treatment	Maintenance of remission
Guslandi *et al.*, 2000	*S. boulardii* in combination with mesalazine	Maintenance of remission more effective than mesalazine alone
Gupta *et al.*, 2000	*Lactobacillus rhamnosus* GG	Improvement of clinical status and barrier integrity
Prantera *et al.*, 2002	*Lactobacillus rhamnosus* GG	Ineffective
Guandalini, 2002	*Lactobacillus rhamnosus* GG	Improvement of clinical status
Bousvaros *et al.*, 2005	*Lactobacillus rhamnosus* GG as adjuvant to standard therapy	Ineffective

Three large trials currently exist regarding the therapeutic effect of *E. coli* Nissle 1917 in maintaining remission of UC. *E. coli* Nissle 1917 was reported to be equally efficacious as mesalazine in preventing relapse of UC (Kruis *et al.*, 1997; Rembacken *et al.*, 1999; Kruis *et al.*, 2001; Kruis *et al.*, 2004). Clinical studies with VSL#3 also resulted in remission maintenance of UC and reduction of active inflammation (Venturi *et al.*, 1999; Fedorak *et al.*, 2003). Furthermore, administration of the yeast *S. boulardii* induces remission in patients with mild to moderate colitis (Guslandi *et al.*, 2003).

In contrast to UC, the effective use of probiotics in CD is not proven. Results from clinical trials regarding CD are mixed. The use of *Lactobacillus rhamnosus* GG alone

(Prantera *et al.*, 2002) and as an adjuvant to standard therapy with e.g. amino-salicylates (Bousvaros *et al.*, 2005) did not show any effect of the probiotic, whereas *Lactobacillus rhamnosus* GG treatment of children with mild to moderate colitis in combination with prednisolone and immunomodulatory drugs indicates that the probiotic has a positive influence on the intestinal barrier integrity (Gupta *et al.*, 2000). Maintenance of prednisolone-induced remission was demonstrated with *E. coli* Nissle 1917 in a randomized, double-blind placebo controlled pilot study (Malchow, 1997). Also *S. boulardii* in combination with mesalazine has a remission maintaining effect (Guslandi *et al.*, 2000).

Although these prospective, randomized clinical trails substantiate the effectiveness of certain probiotic strains in intestinal diseases, further valid clinical trials, incorporating probiotic dose and long-time effect, are necessary.

5.4 *E. coli* Nissle 1917

5.4.1 History

The *E. coli* strain Nissle 1917 (EcN) was originally isolated by the army surgeon Dr. Alfred Nissle in 1917 in the First World War from the feces of a soldier who, in contrast to all of his comrades, did not develop diarrhea during a severe outbreak of shigellosis in the region of Dobrudsha on the Balkan peninsula (Nissle, 1918). EcN is a typical example of a non-pathogenic, commensal *E. coli* isolate, which forms the basis of the probiotic preparation Mutaflor®, used for treatment of various intestinal disorders especially for UC in the phase of remission (Rembacken *et al.*, 1999; Kruis *et al.*, 2004) and chronic constipation (Mollenbrink and Bruckschen, 1994).

5.4.2 Strain-specific characteristics of EcN

Several strain specific characteristics of EcN have been detected so far. EcN of serotype O6:K5:H1 lacks P- and S-fimbriae adhesion determinants but it expresses type 1 and F1C fimbriae, relevant for adhesion and responsible for increased mobility (Blum *et al.*, 1995). The strain exhibits a unique semirough LPS phenotype, responsible for its serum sensitivity (Blum *et al.*, 1995; Grozdanov *et al.*, 2002), and does not produce known toxins (Grozdanov *et al.*, 2002). Furthermore, EcN expresses two microcins (Patzer *et al.*, 2003) and for enhancement of growth, multiplying and success, i.e. its vitality or fitness, this strain possesses six iron-uptake systems (enterobactin, yersiniabactin, aerobactin, salmochelin, ferric dicitrate

transport system, and the *chu* heme transport locus). EcN lacks defined virulence factors (Grozdanov *et al.*, 2004).

All these properties might be advantageous for EcN in competing with other colonic bacteria or adapting to the intestinal situation.

5.5 Mechanisms of action of probiotics

Several mechanisms have been suggested to explain the beneficial effects of probiotics. These proposed mechanisms - summarized in table 4 - include direct effects such as probiotic-luminal organism interactions (e.g. quorum sensing) and interaction with cellular components of the intestinal immune system as well as indirect effects on luminal microorganisms and mucosal immune effector cells mediated through IECs.

Table 4. Proposed mechanisms of action of probiotics.

I. Inhibition of pathogenic bacteria
▪ Inhibition of adherence and invasion ▪ Secretion of bacteriocidal proteins and anti-bacterial peptides (e.g. defensins) ▪ Colonization resistance (occupying an ecologic niche) ▪ Decrease of luminal pH
II. Modulation of intestinal barrier function
▪ Induction of mucin production and secretion ▪ Decrease or normalization of gut permeability
III. Modulation of immune regulation
▪ TLRs ▪ Induction of expression and secretion of antiinflammatory cytokines e.g. IL-10 and TGF-β ▪ Decrease of expression and secretion of proinflammatory cytokines e.g. TNF-α, IFN-γ and IL-12 ▪ Activation of dendritic cells ▪ Activation of neutrophil and T_H1 cell migration

(modified from Sartor, 2004)

Adherence to IECs is the first step for colonization, invasion, and toxin delivery. Until now multiple results have demonstrated the relevance of probiotics - especially of EcN and *Lactobacillus* ssp. - in inhibition of adherence and invasion of pathogenic bacteria as well as prevention of decreased transepithelial resistance (Boudeau *et*

al., 2003; Resta-Lenert and Barrett, 2003; Altenhoefer *et al.*, 2004; Sherman *et al.*, 2005). Probiotic *Lactobacillus* species compete for binding sites with enteric pathogens by adherence to cell surface receptors (Greene and Klaenhammer, 1994). The deficiency of defensins, a group of antimicrobial peptides secreted by IECs and Paneth cells, seems to play an essential role in the pathogenesis of IBDs (Fellermann *et al.*, 2003). EcN may positively stimulate the intestinal innate defense through up-regulation of human beta defensin 2 (HBD2) (Wehkamp *et al.*, 2004). Another proposed mechanism by which probiotics mediate their beneficial effects is the up-regulation and increased secretion of mucins (Mack *et al.*, 1999; Mack *et al.*, 2003). Probiotics also affect the intestinal immune system. The use of various animal models of experimental colitis revealed a decreased expression and secretion of proinflammatory cytokines after probiotic administration (McCarthy *et al.*, 2003; Schultz *et al.*, 2004; Pena *et al.*, 2005). The surface phenotype, cytokine release and immunological functions of dendritic cells can as well be modulated by probiotics (Drakes *et al.*, 2004; Mohamadzadeh *et al.*, 2005).

The current state of research presents multifaceted mechanisms of action initiated by probiotics. Interference with bacterial adherence and modulation of intestinal immune responses seem to be the most important mechanisms in this context.

CHAPTER II

Results

II. Results Part I

1. Background

Since probiotics are widely used as therapeutic agents for gastrointestinal diseases, several studies have investigated the impact of these microorganisms on IECs. Most of the studies have particularly examined the probiotic behavior of lactic acid producing bacteria to prevent invasion and adhesion of pathogenic microorganisms. Until today bacteria of the genera *Bifidobacterium* and *Lactobacillus* are mainly used as probiotics, but in the recent past other microbes also gained interest in this field. Although EcN is used in humans as probiotic treatment for diarrhea and UC, only a few studies have examined the impact of EcN on IECs. *In vitro* investigations revealed that this strain reduces the invasion efficiency of pathogens like *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), *Yersinia enterocolitica* and *Shigella flexneri* (*S. flexneri)* (Altenhoefer *et al.*, 2004). Inhibitory effects of EcN on adhesion and invasion of adherent-invasive *E. coli* were also observed (Boudeau *et al.*, 2003). Few reports are elucidating the therapeutic mechanisms of probiotics using animal colitis models. It has been shown, that administration of EcN reduces proinflammatory cytokine secretion in mouse models of acute and chronic colitis (Schultz *et al.*, 2004). Recently published data revealed that EcN reduced body weight loss and had a positive influence on the disease activity index in mice with acute colitis. Moreover, heat-killed EcN or its genomic DNA itself also reduced the disease burden in a mouse model of acute dextran-sodium sulfate (DSS) induced colitis (Kamada *et al.*, 2005).

Although these data are very promising, the molecular and functional mechanisms responsible for the above reported beneficial effects of EcN on IECs remain to be elucidated.

2. Aims of the study

In order to detect genes potentially responsible for the probiotic effect of EcN at the host level microarray technology was used to analyze the global gene expression profile of IECs. Therefore, Caco-2 cells, a human intestinal epithelial cell line, were cocultured with EcN and their cellular response was compared to that initiated by diarrheagenic *E. coli* stains, the fully sequenced apathogenic K12 *E. coli* isolate MG1655 (Blattner *et al.*, 1997) and the probiotic yeast *S. boulardii*. To elucidate the molecular basics underlying the probiotic effect, data from microarray analyses are expected to provide a subset of genes out of the whole human genome that is specifically regulated after EcN treatment. To accomplish this goal, experiments addressing the following topics were performed:

- Establishment and optimization of a cell coculture system with bacteria followed by identification of an optimal multiplicity of infection (MOI)
- Visualization of bacteria-cell interaction with electron microscopy
- Isolation of eukaryotic RNA followed by cDNA synthesis for microarray hybridization and data analysis
- Realtime RT-PCR for quantitative determination of mRNA levels to verify microarray data
- Expression time kinetics of selected genes
- Impact of EcN viability on expression levels of selected genes
- Quantification of selected proteins
- Confirmation of data obtained from human IECs with a coculture model of primary murine intestinal tissue

3. Results

3.1 *In vitro* model for coculture of human IECs with bacteria

Although several *in vitro* and *in vivo* studies have demonstrated that probiotic-eukaryotic host cell interactions evoke a large number of responses potentially responsible for the positive effects of probiotics, the molecular and functional mechanisms underlying the probiotic effect remain unclear.

For investigations regarding the impact of probiotic EcN on human IECs, a coculture model was established. This *in vitro* model should fulfill the following prerequisites: a coincubation time with viable bacteria or yeasts over a time period that is as long as possible without exhausting the buffer capacity of cell culture media, which would lead to alterations in the gene expression profile. In addition, the model should be easy to handle in order to serve as a read-out system for future experiments.

Caco-2 cells were grown until confluence and cocultured with EcN, the K12 *E. coli* strain MG1655, two enterohemorrhagic *E. coli* strains EHEC126814 and EHEC86-24 (Gunzer *et al.*, 2002) as well as with the probiotic yeast *S. boulardii*. In order to adapt microorganisms to cell culture media, overnight cultures in LB media were diluted 1:100 in prewarmed cell culture media and harvested at late logarithmic phase. By using a low MOI ranging between 0.5 and 1.5, coincubation times of up to 6 hours were possible. A higher MOI resulted in acidification of cell culture media within the 6 hour incubation period. In contrast to bacteria with an *in vitro* doubling time of around 20 minutes, yeasts grew about 4 times slower and reduplicated their numbers every 90 minutes. Therefore, an MOI of 10 was used for coculture experiments with *S. boulardii*. Bacteria and yeast colony forming units (CFU) at the end of the coincubation period were recorded by plating 10-fold dilution series of coculture supernatants onto LB or YPD agar plates.

Interaction between Caco-2 cells and *E. coli* strains or the probiotic yeast was visualized by electron microscopy with IECs grown on collagen I coated coverslips and fixed after coculture (fig. 11). Image I illustrates untreated confluent Caco-2 cells. Cells coincubated with EcN and *E. coli* MG1655 are depicted in image II and III. Image IV shows the formation of pedestals resulting from interaction of EHEC126814 with Caco-2 cells, leading to attaching and effacing lesions typical for EHEC infections (Konowalchuk *et al.*, 1977). Interestingly, EHEC86-24 (image V), which is a

very pathogenic isolate *in vivo* (Gunzer *et al.*, 2002), does not lead to any pedestal formation in the Caco-2 cell coculture system. Image VI illustrates interaction of *S. boulardii* with the cell monolayer and shows budding of yeast cells.

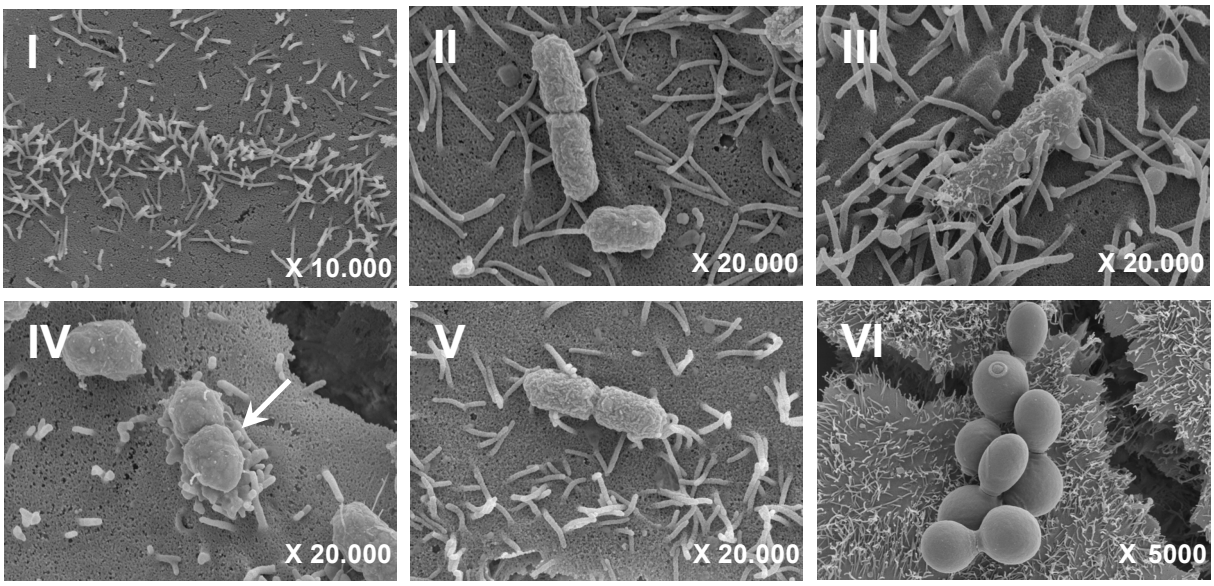

Figure 11. Electron microscopy of coculture experiments.
Confluent Caco-2 cells (I) cocultured with EcN (II), *E. coli* MG1655 (III), EHEC126814 (IV), EHEC86-24 (V) and *S. boulardii* (VI). Arrow indicates pedestal formation.

3.2 Gene expression profile of human IECs cocultured with probiotic or pathogenic microorganisms

In order to obtain a comprehensive overview of the gene expression profile of host IECs in response to probiotic EcN, differential gene expression analysis was performed using Affymetrix HG_U133A arrays. To this end, Caco-2 cells were cocultured with EcN, *E. coli* MG1655, EHEC126814, EHEC86-24 and *S. boulardii* for 6 hours; RNA was prepared from 0.4 – 1x10^7 cells, converted to cDNA, transcribed to biotinylated cRNA and subjected to differential gene expression analysis with the microarrays. Quality of total RNA and cRNA synthesis was controlled by running all samples on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) (fig. 12).

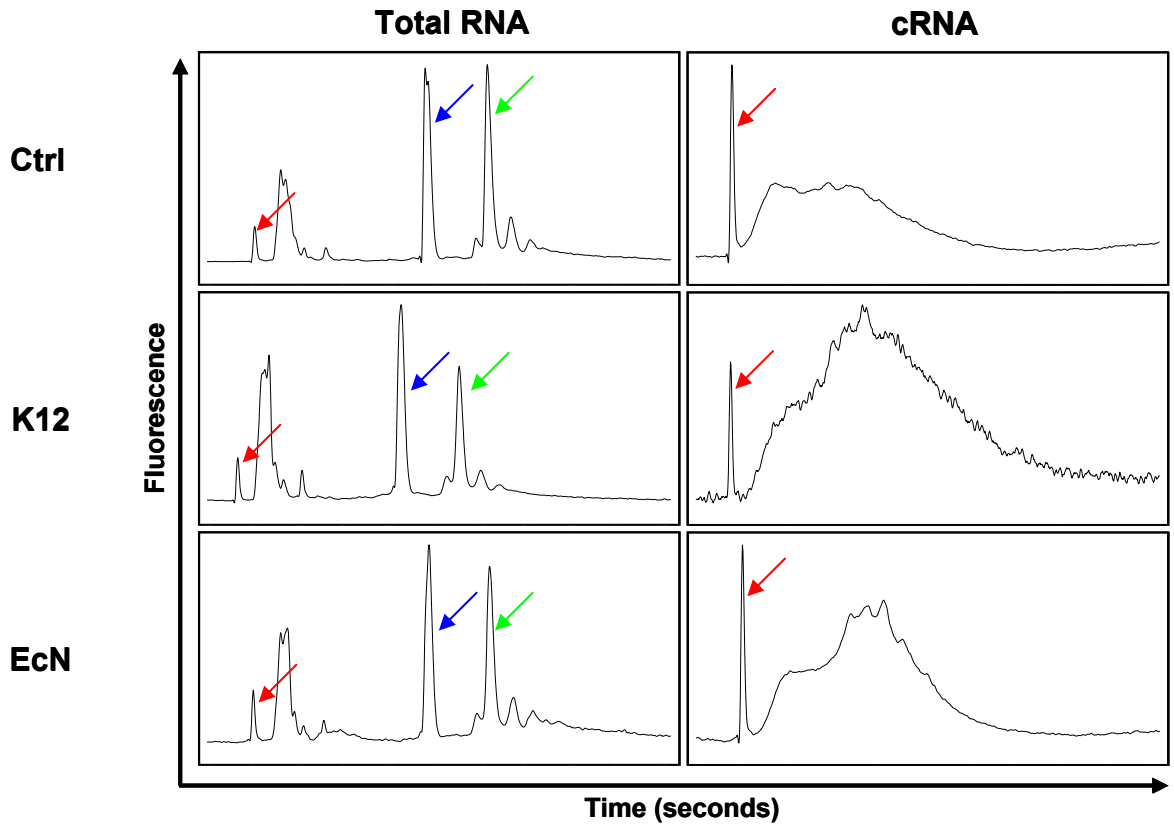

Figure 12. Agilent Bioanalyzer electropherograms.
RNA6000 lab chips were loaded with gel-dye mix, RNA6000 Nano Marker, RNA6000 ladder and aliquots of total RNA as well as cRNA from Caco-2 cells cocultured with *E. coli* MG1655 (K12) and EcN. Untreated Caco-2 cells served as control (Ctrl). If RNA fragments are detectable as peaks, RNA is not degraded. The height of the peaks correlates positively to the amount of RNA isolated. Distribution of the cRNA curve along the x-axis demonstrates successful cRNA synthesis. Red arrows indicate marker, blue arrows 18S RNA and green arrows 28S RNA.

With Affymetrix HG_U133A arrays the expression level of over 22,000 human transcripts and variants can be analyzed. Furthermore, every gene analyzed is represented by eleven independent probe pairs which establish the basis for statistical analysis of the respective signals. Consequently, only those genes that are reproducibly regulated are included in the final evaluation. Genes are considered to be regulated with an expression level (average fold change) that is at least 2-fold increased or 2-fold decreased compared to non-treated Caco-2 cells.

Among the 22,000 genes and expressed sequence tags present on Affymetrix HG_U133A arrays, 138 sequences were identified as expressed differentially upon treatment of confluent Caco-2 cells with EcN. Due to some genes being repeatedly present on the array, a total of 126 individual genes were finally found to be regulated, assigned to a wide variety of gene classes (fig. 13). 17% of the coding sequences belonged to signaling pathways. The remainder of the genes encoded for molecules involved in protein folding - and biosynthesis (10%), transcription and

translation (10%), transport (8%), metabolism (6%) as well as energy pathways (5%), immune response (4%) proliferation (3%), differentiation (1%) and apoptosis (1%).

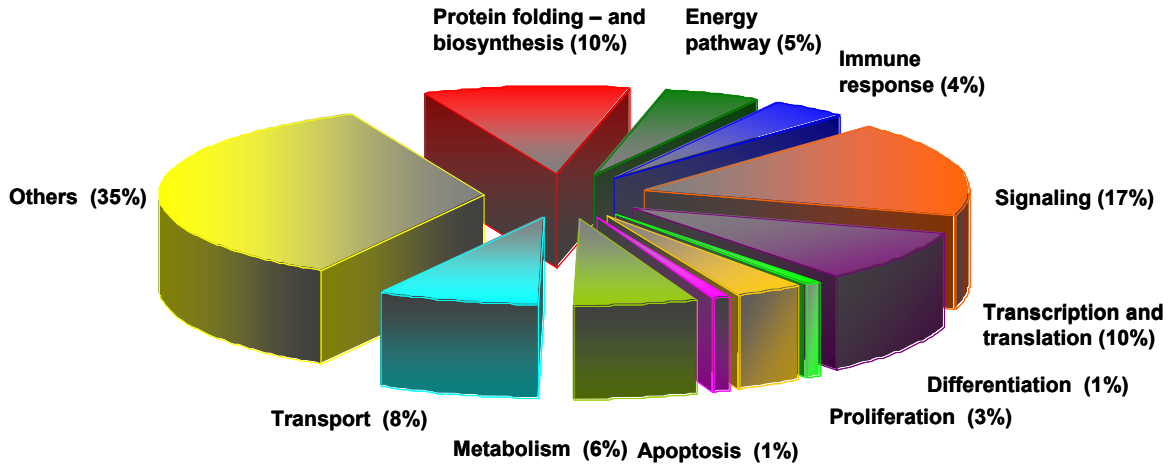

Figure 13. Classification of genes regulated in confluent Caco-2 cells after coculture with EcN for 6 hours.
Analysis of microarray data from two independent experiments resulted in 126 genes being regulated. These genes could be assigned to different classes according to their involvement in biological processes (percentage by number of genes per class).

A larger part of them, like genes coding for hypothetical proteins and unannotated ORFs, could not be allocated to one of the classes and was therefore assigned as others (35%).

In order to classify the expression of genes specific for EcN, the signal intensities of these 126 genes regulated after EcN treatment were compared to the signal intensities of non-treated Caco-2 cells and to those treated with *E. coli* MG1655, EHEC126814, EHEC86-24 and *S. boulardii*. These co-regulated genes were combined in a hierarchical clustering analysis summarized in figure 14. Surprisingly, treatment of Caco-2 cells with EcN and pathogenic EHEC126814 resulted in an expression profile with more than 50% overlap (fig. 14). To end up with a subset of genes specifically regulated by EcN in comparison to the other microorganisms investigated, k-means cluster analyses were performed subsequently.

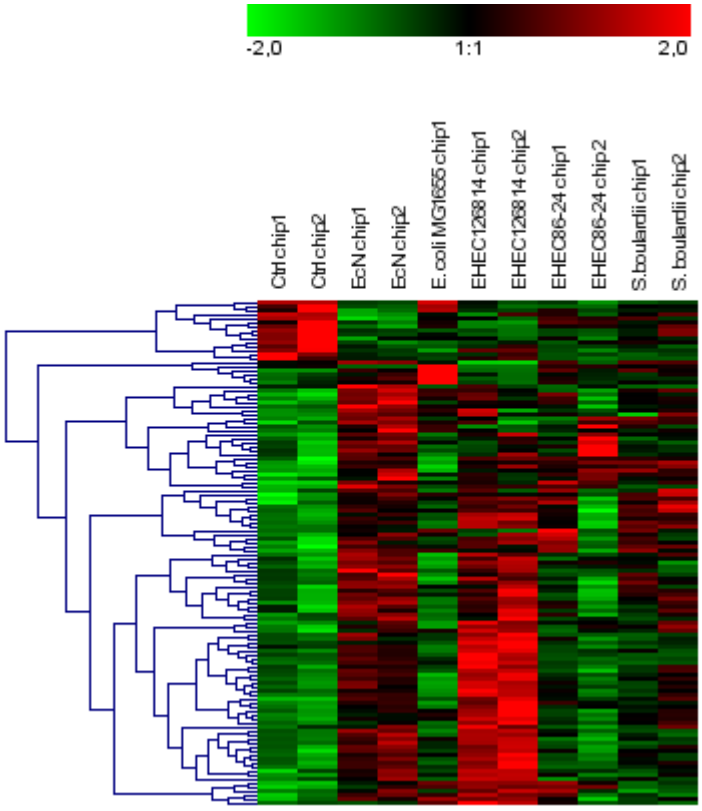

Figure 14. Hierarchical clustering of gene signal intensities.
The signal intensities of the 126 genes specifically regulated in Caco-2 cells after EcN treatment were compared to the signal intensities after treatment with *E. coli* MG1655, EHEC126814, EHEC86-24 and *S. boulardii*. Red indicates induction of gene expression, green indicates repression. The brighter the color, the stronger the factor of gene regulation (+2: bright red; -2: bright green). Black indicates no changes. Inclusion into this heat map required at least a 2-fold difference in gene expression of Caco-2 cells cocultured with EcN compared to control cells. Two independent experiments were performed (chip1, chip2).

These analyses produced four clusters (A-D) as depicted in figure 15. The clusters combined signal intensities of genes either specifically down- or up-regulated in Caco-2 cells after EcN treatment. Genes with a very low basal expression level in control cells and a specific up-regulation after coculture with microorganisms were summarized in cluster A. In contrast, cluster B shows genes with very high signal intensities in control cells that were down-regulated after bacterial treatment. Genes strongly up-regulated in response to EcN and EHEC126814, like the ones coding for proinflammatory chemokines CXCL2 (MIP-2α) and CXCL3 (MIP-2β), are illustrated in cluster C. Genes specifically regulated in human IECs in response to coculture with EcN are summarized in cluster D, the most important cluster regarding the aim of this study. Interestingly, one of these genes codes for proinflammatory cytokine CCL2 (MCP-1).

Selected genes of each cluster with at least a 2-fold regulation in one of the two experiments were choosen on the basis of both, prominent changes in their gene expression profiles and their putative biological implication regarding a probiotic effect. These genes are summarized in table 5.

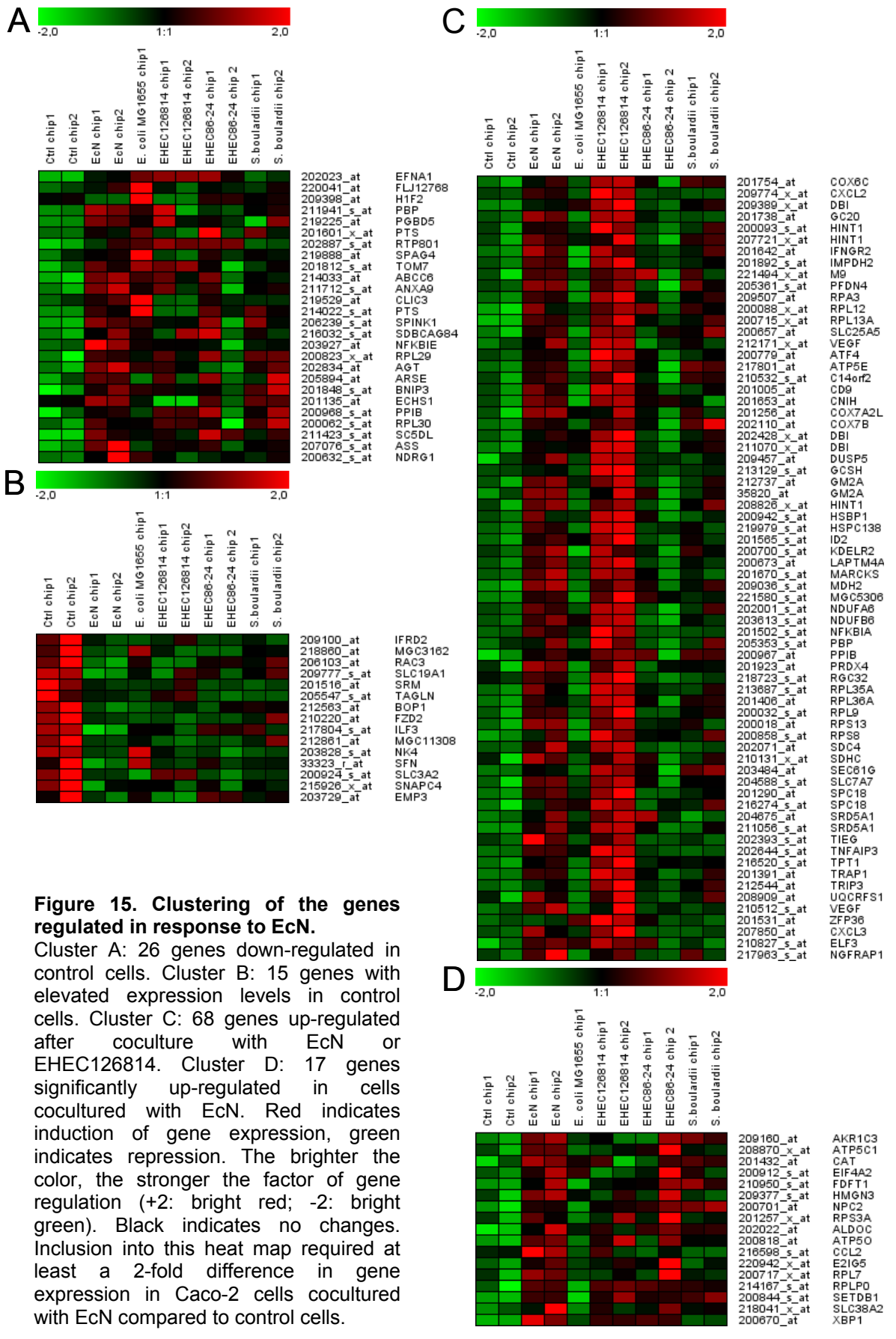

Figure 15. Clustering of the genes regulated in response to EcN.
Cluster A: 26 genes down-regulated in control cells. Cluster B: 15 genes with elevated expression levels in control cells. Cluster C: 68 genes up-regulated after coculture with EcN or EHEC126814. Cluster D: 17 genes significantly up-regulated in cells cocultured with EcN. Red indicates induction of gene expression, green indicates repression. The brighter the color, the stronger the factor of gene regulation (+2: bright red; -2: bright green). Black indicates no changes. Inclusion into this heat map required at least a 2-fold difference in gene expression in Caco-2 cells cocultured with EcN compared to control cells.

Table 5. Differential gene expression of Caco-2 cells cocultured with EcN.

Functional category	Gene symbol	GB Acc. No.[b]	Fc EcN/Ctrl		Fc K12/Ctrl	Fc E126/Ctrl		Fc E86/Ctrl		Fc Sb/Ctrl		Description and/or putative function
			Chip 1	Chip 2	Chip 1	Chip 1	Chip 2	Chip 1	Chip 2	Chip 1	Chip 2	
Immune response	MCP-1 (CCL2)	S69738.1	11.1	3.1	-	-	-	-	-	-	-	Enhances the inflammatory response, up-regulated by IL1α and TNFα
	CD9	NM_001769.1	1.7	3.6	-2.6	2.5	4.8	-	-	-	-	Involved in platelet activation and aggregation
	MIP-2α (CXCL2)	M57731.1	4.4	8.1	-	12.9	33.4	-	-	-	-	Produced by activated monocytes, expressed at inflammation sites, up-regulated by IL1α and TNFα
	MIP-2β (CXCL3)	NM_002090.1	6.5	10.4	-	6.5	27.7	-	-	-	-	Plays a role in inflammation, autocrine effect on endothelial cells
	NK4	NM_004221.1	-1.7	-2.5	-	-	-	-2.0	-3.3	-2.9	-3.1	May play a role in lymphocyte acitivation, angiogenesis inhibitor, putative therapeutic agent for gastric cancer
Signaling	AGT	NM_000029.1	1.7	2.6	-	-	-	-	-	1.5	2.1	Serine protease inhibitor
	CNIH	NM_005776.1	2.6	6.9	-	2.1	13.8	-	-	-	-	EGF-signaling in oocyte
	DUSP5	U16996.1	13.1	3.4	-	-	-	-	-	-	-	Hydrolase, negative feedback role in IL-2 signaling
	ID2	NM_002166.1	2.8	4.2	2.1	3.4	8.2	-	-	2.2	3.1	Essential for constituting the intestinal mucosal barrier
	IFNGR2	NM_005534.1	1.8	2.4	-	2.6	2.5	-	-	-	-	Protein translocator
	NFκBIA	AI078167	6.3	3.3	2.9	9.9	5.4	-	-	-	-	Downregulation of NF-κB activity, contributed in CD
	NFκBIE	NM_004556.1	3.3	2.1	-	-	-	-	-	-	-	Inhibits NF-κB
	PRDX4	NM_006406.1	1.7	3.6	-	1.9	3.8	-	-	-	-	Regulates the activation of NF-κB by modulation of IκBα phosphorylation
	TNFαIP3	NM_006290.1	4.3	3.3	2.4	6.2	3.9	-	-	-	-	Interacts with NAF1 and inhibits TNF-induced NF-κB dependent gene expression
	SFN	X57348	-1.6	-2.2	-	-	-	-	-	-	-	Exonuclease specific for small oligoribonucleotides
Transcription and translation	ELF3	U73844.1	3.3	1.8	3.1	3.7	2.1	-	-	-	-	Epithelial specific ets transcription factor, regulates MIP3α expression which is NF-κB dependent
	ILF3	BC003086.1	-4.3	-2.0	-2.2	-	-	-	-	-	-	May faciliate dsRNA-regulated gene expression
	XBP1	NM_005080.1	2.5	2.7	-	2.1	3.3	-	-	-	-	May act as transcription factor in B-cells
Apoptosis	BNIP3	U15174.1	3.0	2.7	3.0	2.8	1.7	-	-	2.9	2.5	Apoptosis inducing protein, binding to BCL2
Differentiation	NDRG1	NM_006096.1	2.4	6.8	2.4	-	-	-	-	-	-	Growth inhibitory role
Proliferation	EMP3	NM_001425.1	-2.2	-4.4	-	-3.2	-4.5	-	-	-	-	Probably involved in cell proliferation
	VEGF	H95344	2.1	2.2	-	3.6	4.8	-	-	-	-	Induced by mek1, role of vascular endothelial growth factor in IBD

Fc; Fold change is the factor of mRNA regulation from bacteria-treated cells and non-treated cells according to the signal values themselves and to the change value from Affymetrix analysis software. Minus fold change values indicate genes with decreased transcription. Results have been performed by two independent hybridization experiments (Chip1, Chip2); [1] GB Acc. No. indicates GenBank accession number; -, not regulated; K12, *E. coli* MG1655; E126, EHEC126814; E86, EHEC86-24; Sb, *S. boulardii*

Significantly elevated mRNA levels were found for dual-specificity phosphatase 5 (DUSP5), chemoattractant protein-1 ligand 2 (MCP-1), macrophage inflammatory protein-2 alpha (MIP-2α), macrophage inflammatory protein-2 beta (MIP-2β), tumor necrosis factor alpha-induced protein 3 (TNFαIP3) and ets domain transcription factor ELF3.

DUSP5 is a VH1-like enzyme that hydrolyses nuclear substrates phosphorylated on both tyrosine and serine/threonine residues, which has a potential role in deactivation of mitogen - or stress-activated protein kinases and plays a negative feedback role in IL-2 signaling (Kovanen *et al.*, 2003). Interestingly, MCP-1 gene expression was also up-regulated after EcN treatment of Caco-2 cells. MCP-1 is produced by many cells, including epithelial, endothelial and mast cells as well as by tumor cells, and shows chemotactic activity for monocytes, basophils, natural killer (NK) cells and T lymphocytes during inflammation. MCP-1 induces the release of specific enzymes of the target cells (Jiang *et al.*, 1992; Dahinden *et al.*, 1994; Taub *et al.*, 1995; Loetscher *et al.*, 1996). Gene expression for MIP-2α and MIP-2β, two additional proteins involved in inflammatory processes, was found to be up-regulated in array experiments. Both genes encode for chemokines that attract neutrophils. MIP-2α is produced by activated monocytes and is up-regulated after exposure to the proinflammatory cytokines IL-1α and TNF-α (Yamagami *et al.*, 2003). Up-regulated gene expression was also detected for TNFαIP3, a zinc finger protein, which is encoded by an immediate early response gene, and acts as a potent inhibitor of NF-κB-dependent gene regulation. It has been speculated that TNFαIP3 substrates are positive regulators of NF-κB signaling unique to TNF-α -activated pathways (Wertz *et al.*, 2004). However, other findings suggest that TNFαIP3 may function as a negative regulator of TLR-mediated inflammatory responses in human airway epithelial cells, thereby protecting the host against harmful overresponses to pathogens (Gon *et al.*, 2004). A further gene up-regulated after coculture of Caco-2 cells with EcN encodes ets domain transcription factor ELF3, a critical regulator of epithelial cell differentiation (Oettgen *et al.*, 1997). The gene encoding vascular endothelial growth factor (VEGF) was up-regulated as well. VEGF is active in angiogenesis, vasculogenesis and endothelial cell growth. An increased mucosal secretion of VEGF could be observed in patients with collagenous colitis (Taha *et al.*, 2004), but VEGF expression is absent in patients with CD and weakly positive in individuals with UC (Giatromanolaki *et al.*, 2003). Yet another gene, up-regulated by

EcN treatment, is coding for human nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha (NFκBIA). NFκBIA inhibits the action of NF-κB by forming a heterodimer with NF-κB, thus preventing its translocation to the nucleus (Ito *et al.*, 1995). Downregulation of gene expression was observed for epithelial membrane protein 3 (EMP3). EMP3 belongs to the peripheral myelin protein 22 family, comprising small hydrophobic membrane proteins and is highly expressed in peripheral blood leukocytes, ovaries, intestine and various embryonic tissues (Taylor and Suter *et al.*, 1996; Jetten and Suter *et al.*, 2000). Further genes up-regulated by EcN in Caco-2 cells encode N-myc downstream regulated gene 1 (NDRG1), peroxiredoxin 4 (PRDX4), BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), inhibitor of DNA binding 2 (ID2) and antigen CD9. Genes of stratifin (SFN) and interleukin enhancer binding factor 3 (ILF3) were found to be down-regulated. NDRG1 is strongly up-regulated in differentiation of colon epithelial cell lines (van Belzen *et al.*, 1997). Its protein function in humans is still unknown. PRDX4 is one of six genes belonging to the peroxiredoxin-family that are highly conserved in eukaryotes and prokaryotes and are ubiquitously expressed. Peroxiredoxins exhibit thioredoxin-dependent peroxidase activity and have been implicated in a number of other cellular functions such as cell proliferation and differentiation. PRDX4 plays a regulatory role in the activation of NF-κB (Zhang *et al.*, 2004). Proapototic BNIP3 gene expression is increased in many cell types during hypoxia. Forced overexpression of BNIP3 induces cell death (Kothari *et al.*, 2003). ID2, an inhibitor of basic helix-loop-helix transcription factors, regulates cell differentiation. Id2-/- mice exhibit a variety of phenotypes in the immune system. Interestingly, experiments with these mice revealed among others that the Id2 gene, selectively expressed in all T cell subsets in the small intestinal intraepithelial lymphocytes, is essential for constituting the intestinal mucosal barrier (Kim *et al.*, 2004). The tetraspanine transmembrane protein CD9 is associated with platelet activation (Worthington *et al.*, 1990; Qi *et al.*, 1996) and is expressed on mouse intestinal epithelial exosomes linking to the immune system (Van Niel *et al.*, 2003). Stratifin, also known as 14-3-3 sigma, is expressed primarily in epithelial cells and appears to play a unique role in the cellular response to DNA damage and in human oncogenesis. The biological and structural basis for the stratifin-specific functions is unknown (Wilker *et al.*, 2005).
Surprisingly, microarray data revealed that 68 of the 126 genes (fig. 15 C) were regulated both in response to probiotic EcN and pathogenic EHEC126814. Genes

coding for proinflammatory chemokines MIP-2α and MIP-2 β were included in this group. A subset of 17 genes is specifically up-regulated by EcN, comprising the gene encoding proinflammatory chemokine MCP-1 (fig. 15 D).

3.3 Realtime RT-PCR to confirm data obtained from gene expression analysis

To confirm results obtained from global gene expression profiling, regulation of selected genes was quantified by realtime RT-PCR analyses.

Array data for gene regulation of MCP-1, MIP-2α, MIP-2β, DUSP5, ELF3 and NFκBIA were verified by realtime RT-PCR (fig. 16a).

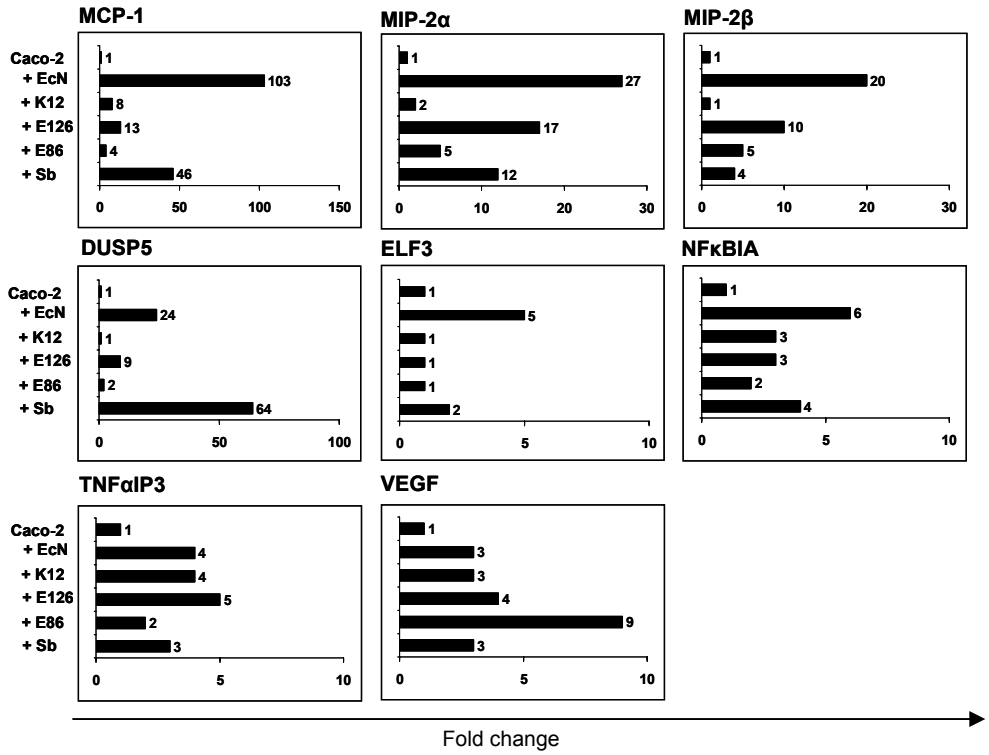

Figure 16a. Validation of mRNA expression levels of Caco-2 cells by quantitative realtime RT-PCR.

After 6 hours of coincubation of Caco-2 cells with EcN (+ EcN), *E. coli* MG1655 (+ K12), EHEC126814 (+ E126), EHEC86-24 (+ E86) or *S. boulardii* (+ Sb), total RNA was isolated, reversely transcribed, and relative mRNA expression levels for selected genes and the housekeeping gene RPS9 were analyzed in duplicate realtime RT-PCR assays. Relative mRNA amounts were normalized with respect to expression levels of untreated Caco-2 cells (fold change = 1). The figure is representative of two independent experiments. MCP-1 indicates chemoattractant protein-1 ligand 2; MIP-2α, macrophage inflammatory protein-2 alpha; MIP-2β, macrophage inflammatory protein-2 beta; DUSP5, dual specificity phosphatase 5; ELF3, E74-like factor 3 (ets domain transcription factor, epithelial-specific), NFκBIA, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha; TNFαIP3, tumor necrosis factor alpha-induced protein 3 and VEGF, vascular endothelial growth factor.

The most impressive up-regulation of mRNA expression was detected for MCP-1. There was a 103-fold increase, compared to mRNA levels elevated only 8-fold after treatment with *E. coli* MG1655, 13-fold with EHEC126814, 4-fold with EHEC86-24 and 46-fold with *S. boulardii*. Additionally, gene expression levels of MIP-2α and MIP-2β were significantly elevated in Caco-2 cells treated with EcN in comparison to control strains. It should be emphasized that MCP-1, MIP-2α and MIP-2β gene expression levels were decreased after EHEC126814 treatment compared to EcN treatment. In addition, realtime RT-PCR analysis revealed an EcN specific up-regulation of NFκBIA and ELF3 gene expression. However, this was not the case for DUSP5, TNFαIP3 and VEGF. In contrast to the above mentioned genes, for which the expression data obtained from array analysis were confirmed by realtime RT-PCR, the expression changes of the following genes could not be verified (fig. 16b).

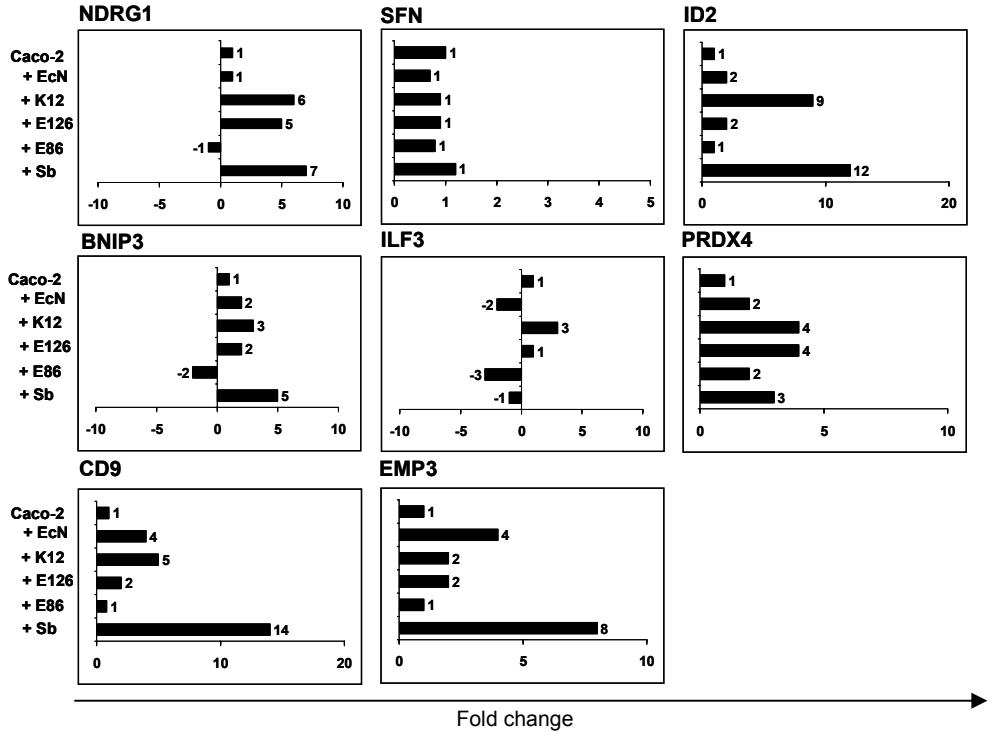

Figure 16b. Validation of mRNA expression levels of Caco-2 cells by quantitative realtime RT-PCR.
After 6 hours of coincubation of Caco-2 cells with EcN (+ EcN), *E. coli* MG1655 (+ K12), EHEC126814 (+ E126), EHEC86-24 (+ E86) or *S. boulardii* (+ Sb), total RNA was isolated, reversely transcribed, and relative mRNA expression levels for selected genes and the housekeeping gene RPS9 (as internal control) were analyzed in duplicate realtime RT-PCR assays. Relative mRNA amounts were normalized with respect to expression levels of untreated Caco-2 cells (fold change = 1). The figure is representative of two independent experiments. NDRG1 indicates N-myc downstream regulated gene 1; PRDX4, peroxiredoxin 4; BNIP3, BCL2/adenovirus E1B 19kDa interacting protein 3; ID2, inhibitor of DNA binding 2; CD9, CD9 antigen; SFN, stratifin and ILF3, interleukin enhancer binding factor 3.

Furthermore, no EcN specific regulation could be detected for any of these genes (fig. 16b).

Immune and inflammatory responses in IECs often involve the transcription factor NF-κB. This DNA binding protein is the transcriptional effector of a conserved regulatory pathway that is activated by a multiplicity of proinflammatory stimuli and is required for the *de novo* synthesis of numerous proinflammatory molecules and other molecules critical for normal immuno-inflammatory function (Ghosh *et al.*, 1998). Many of the genes essential for cellular immune responses and inflammation, activated in IECs after contact with bacterial pathogens (e.g. by invasion), are target genes of the transcription factor NF-κB. NF-κB was suggested to be the major transcriptional regulator of MCP-1 (Collins *et al.*, 1995). Array analysis revealed no regulation of NF-κB gene expression in response to EcN treatment. But, with respect to the significant up-regulation of MCP-1 gene expression, realtime RT-PCR of p105/p50 (NF-κB1) and p49/p100 (NF-κB2) was performed. Slightly increased mRNA levels could be detected for NF-κB1 after coculture with EcN. In contrast NF-κB2 was not specifically regulated by EcN (fig. 17).

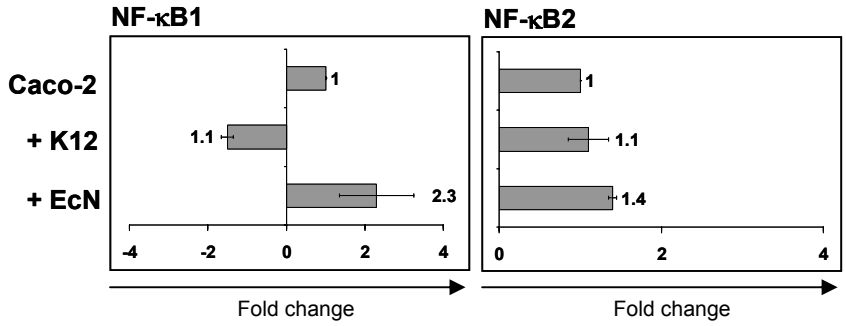

Figure 17. NFκB1 and NFκB2 gene expression in Caco-2 cells.
Caco-2 cells were cocultured with *E. coli* MG1655 (+ K12) or EcN (+ EcN) for 6 hours, respectively. RNA was isolated, reversely transcribed and relative mRNA levels were determined by realtime RT-PCR. Data are presented as mean of two independent experiments. NF–κB1 indicates p105/p50 and NF-κB2 p49/p100.

3.4 EcN specific up-regulation of MCP-1 and MIP-2α gene expression is not a Caco-2 cell specific phenomenon

To exclude that the EcN specific up-regulation of MCP-1 and MIP-2α was a feature specific for Caco-2 cells, coculture experiments were performed with Lovo cells, a second human colon adenocarcinoma cell line. Lovo cells were grown until confluence as described for Caco-2 cells and cocultured for 6 hours with EcN or *E. coli* MG1655 using an MOI of 1, respectively. As already documented for Caco-2 cells, EcN again induced a strong up-regulation of these two proinflammatory genes. The gene expression level of MCP-1 was 76-fold increased compared to control cells, whereas treatment with *E. coli* MG1655 only resulted in a fold change of 2 (fig. 18).

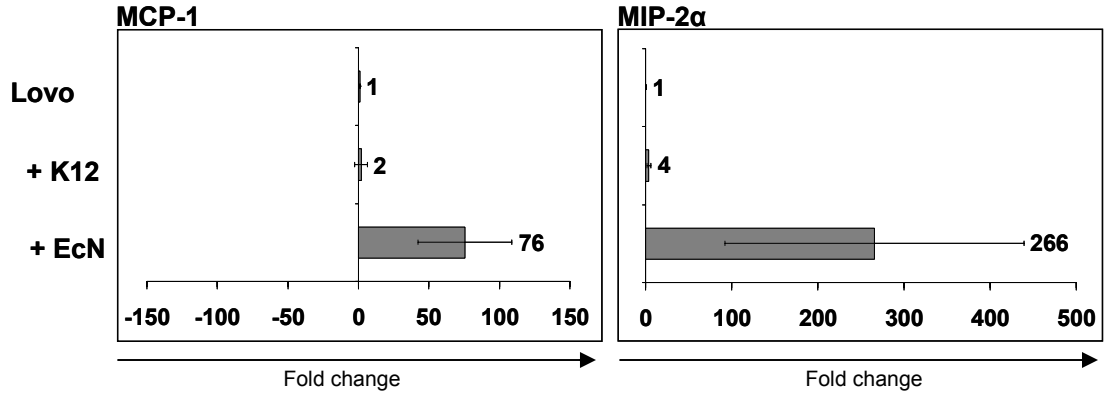

Figure 18. MCP-1 and MIP-2α gene expression in Lovo cells.
Validation of mRNA expression levels of Lovo cells after 6 hours coculture with *E. coli* MG1655 (+ K12) or EcN (+ EcN). Relative mRNA expression levels for MCP-1, MIP-2α and RPS-9 (as internal control) were analyzed in duplicate realtime RT-PCR assays. Relative mRNA amounts were normalized with respect to expression levels of untreated Lovo cells (fold change = 1). Data are presented as mean of three independent experiments. MCP-1 indicates chemoattractant protein-1 ligand 2; MIP-2α, macrophage inflammatory protein-2 alpha.

In addition, MIP-2α gene expression was 266-fold up-regulated after treatment with EcN in comparison to a 4-fold elevation mediated by *E. coli* MG1655. Results obtained from conventional RT-PCR also revealed an EcN specific induction of MIP-2β mRNA expression (fig. 19).

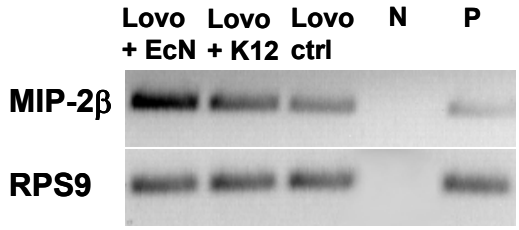

Figure 19. RT-PCR of MIP-2β cDNA in Lovo cells cocultured with EcN or *E. coli* MG1655.
Lovo cells were cocultured with EcN (+ EcN) or *E. coli* MG1655 (+ K12) for 6 hours, respectively. RNA was isolated, reversely transcribed and RT-PCR performed. Caco-2 cells cocultured with *E. coli* MG1655 served as a positive control (P) and sterile H_2O as negative control (N). MIP-2β, macrophage inflammatory protein-2 beta; RPS9, ribosomal protein 9.

Thus, up-regulation of MCP-1, MIP-2α and MIP-2β gene expression after treatment with EcN was not a feature of one cell line but rather a general EcN specific effect on human IECs.

3.5 Gene expression of MCP-1, MIP-2α and MIP-2β is time-dependent

Previous data revealed that EcN specifically up-regulated gene expression of the proinflammatory molecules MCP-1, MIP-2α and MIP-2β as well as of the inhibitor of NFκB following Caco-2 coculture. To address the question, whether this gene expression pattern is time-dependent, time kinetics of the above mentioned genes were performed.

Therefore, bacteria containing supernatants of cell coculture were collected after 6 hours, centrifuged and the bacterial pellets were redissolved in fresh cell culture media. Extended treatment of Caco-2 cells with EcN or *E. coli* MG1655 for a total of 24 and 48 hours revealed that EcN mediated gene expression of MCP-1 peaked after 6 hours and returned to base levels after 24 hours (fig. 20). In contrast, expression of MIP-2α and MIP-2β increased further and reached maximum values after 24 hours. However, MIP-2α and MIP-2β gene expression constantly decreased over the next 24 hours. No signifcant changes in gene regulation after 24 and 48 hours were detected for NFκBIA.

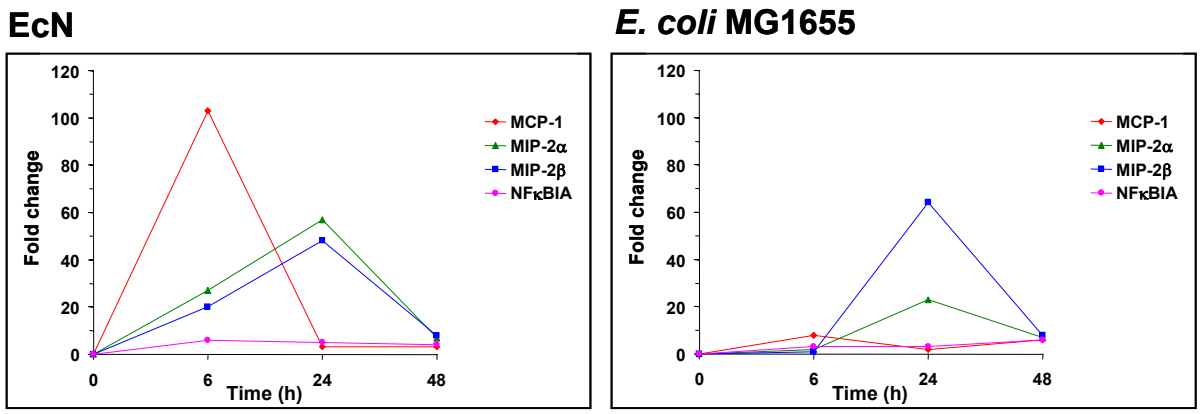

Figure 20. Time-dependency of the gene expression profile of Caco-2 cells treated with EcN. Caco-2 cells were cocultured with EcN and *E. coli* MG1655 for 6, 24 and 48 hours. Gene expression at indicated time points was measured by realtime RT-PCR.

E. coli MG1655 influenced the expression of most of the selected genes just slightly at any time, but revealed a peak for MIP-2β expression after 24 hours, similar to EcN (fig. 20).

3.6 EcN induced gene expression of MIP-2α and MIP-2β is not dependent on viable bacteria

As indicated by several publications, the viability of bacteria plays an essential role for responses resulting from interaction between host cells and microbes. Viable probiotic *Lactobacillus casei* stimulates the intestinal mucosal immune system to a greater extent than nonviable bacteria (Galdeano and Perdignon, 2004). Another study revealed the dependence of the suppressive effect on EHEC internalization by *Lactobacillus rhamnosus* on the viability of the probiotic (Hirano *et al.*, 2003). Due to the impact of the bacterial viability in the context of their effectiveness to excite a host response, it was important to investigate, whether the observed EcN specific expression of selected genes was merely a result of contact between bacterial surfaces and Caco-2 cells and additionally, whether the secretion of active bacterial metabolites was required for the EcN specific induction of the selected genes. Hence, Caco-2 cells were either cocultured with inactivated bacteria or bacteria conditioned media (CM). For inactivation of bacteria, bacterial suspensions were centrifuged, the bacterial pellet was fixed with 4% paraformaldehyde, washed extensively and resuspended in culture media. CM was collected from bacterial suspensions. Therefore, bacterial overday cultures were diluted with cell culture media to $1x10^8$ CFU/ml, centrifuged and the supernatant was sterile filtrated.

The treatment of the IECs with inactivated bacteria revealed no longer the EcN specific up-regulation of MCP-1 and NFκBIA since the expression changes were similar or lower compared to cells treated with inactivated *E. coli* MG1655 (tab. 6). Hence, EcN specific induction of expression of these genes is apparently dependent on live bacteria. In contrast, the EcN specific increase of MIP-2α and MIP-2β as well as of ELF3 mRNA expression seems to be independent on the viability of the bacteria. The expression levels of these genes are still increased after coculture with inactivated EcN compared to control bacteria. In contrast to coculture with inactivated bacteria, the treatment of Caco-2 cells with bacteria CM did not lead to an EcN specific regulation of any of the selected genes (tab. 6).

Table 6. EcN specific expression of selected genes in Caco-2 cells cocultured with inactivated bacteria or bacteria CM for 6 hours.

Gene	Inactivated EcN	EcN CM
ELF3	**(+)**	no EcN specific gene regulation
MCP-1	no EcN specific gene regulation	no EcN specific gene regulation
MIP-2α	**(+++)**	no EcN specific gene regulation
MIP-2β	**(++)**	no EcN specific gene regulation
NFκBIA	no EcN specific gene regulation	no EcN specific gene regulation

Gene expression was analyzed by realtime RT-PCR and differences of fold changes for EcN and *E. coli* MG1655 were compared. EcN specific fold change of Caco-2 cell gene expression (+) between 0.5 and 2, (++) between 2 and 10 and (+++) > 10.

Thus, EcN apparently does not secrete active metabolites responsible for the specific gene expression observed.

To summarize, data from array analysis revealed an EcN specific increase of MCP-1 gene expression at mRNA level and furthermore up-regulation of MIP-2α and MIP-2β after treatment of Caco-2 cells with EcN or EHEC126814. These very interesting results were confirmed by realtime RT-PCR and indicate a temporary EcN specific up-regulation of these genes encoding proinflammatory molecules, not caused by secreted components and, in case of MCP-1, dependent on bacterial viability. Since

this observation is surprising and unexpected at first sight regarding the probiotic nature of EcN, the following experiments focused on these molecules.

3.7 Detection of MCP-1 and IP-10 in Caco-2 coculture supernatants by CBA analysis

Coculture of Caco-2 cells with EcN resulted in up-regulation of MCP-1 at mRNA level. Consequently, it was necessary to determine, whether MCP-1 expression was also increased at protein level. Therefore, supernatants from Caco-2 cells cocultured with EcN and *E. coli* MG1655 for 6 hours were analyzed for several cytokines using the Cytometric Bead Array (CBA) Human Chemokine Kit I (BD Biosciences, Heidelberg, Germany).

Coculture with EcN strongly increased release of MCP-1 protein up to 332 pg/ml compared to untreated Caco-2 cells and those treated with *E. coli* MG1655 with an MCP-1 protein concentration of 78 pg/ml and 60 pg/ml, respectively (fig. 21).

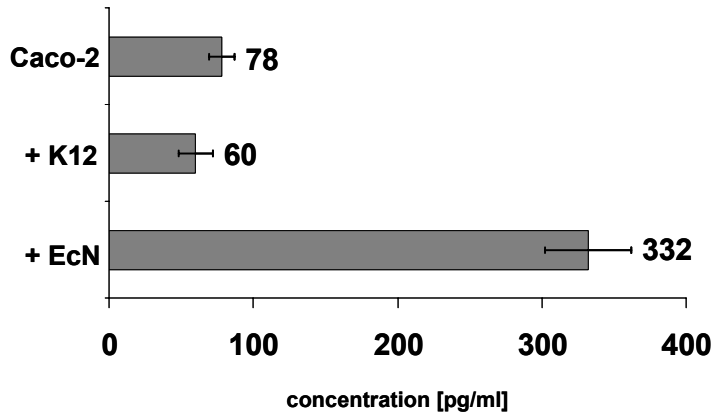

Figure 21. MCP-1 secretion of Caco-2 cells after treatment with EcN.
Caco-2 cells were cocultured with *E. coli* MG1655 (+ K12) and EcN (+ EcN) for 6 hours. Culture supernatants were analyzed for several cytokines using the CBA Human Chemokine Kit I from BD Bioscience. Cytokine quantity is depicted as pg/ml. Data are presented as mean from two independent experiments.

Interestingly, although IP-10, a chemokine that attracts monocytes and T lymphocytes and promotes T_H1 immunity, was not detectable at mRNA level by microarray analysis and RT-PCR, EcN treatment caused an increased IP-10 protein secretion in the cell culture supernatants (fig. 22).

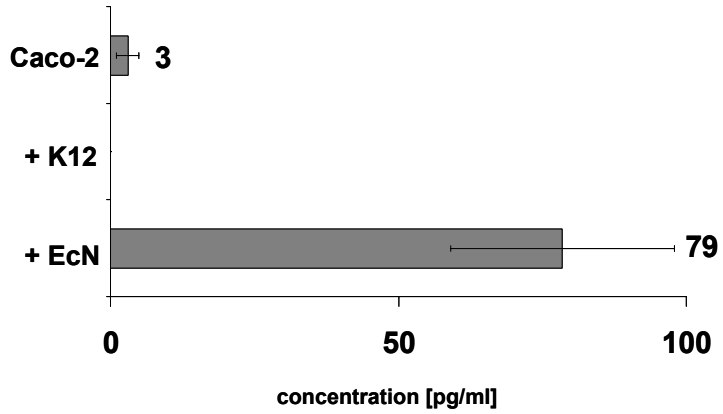

Figure 22. IP-10 secretion of Caco-2 cells after treatment with EcN.
Caco-2 cells were cocultured with *E. coli* MG1655 (+ K12) and EcN (+ EcN) for 6 hours. Culture supernatants were analyzed for several cytokines using the CBA Human Chemokine Kit I from BD Bioscience. Cytokine quantity is depicted as pg/ml. Data are presented as mean from two independent experiments.

3.8 MCP-1 gene expression is up-regulated in small intestine after EcN treatment

Previous results obtained with human intestinal epithelial cell lines rose the question, whether these data could be complemented with primary mouse IECs.

Therefore, the effect of EcN on MCP-1 expression in freshly isolated mouse intestinal epithelial layers was examined. Cima *et al.* described an efficient method for the differential isolation of IECs along the villus-crypt axis of the small intestine (Cima *et al.*, 2004). Using a modification of this method, pieces of small intestine were cocultured with $1x10^6$ CFU EcN or *E. coli* MG1655 to analyze the impact of EcN on mouse primary tissue culture. The relatively low CFU compared to coculture with human IECs was used to prevent rapid acidification of the cell culture media due to growth of the test strain together with the resident flora from tissue pieces. Several reasons argue for using small intestinal tissue for these experiments. Since direct contact between bacteria and IECs was a condition required to confirm data obtained in cell coculture with human IECs, a smooth removal of the mucus layer was necessary. This is ensured for small intestine as it has a thinner mucus layer than the colon (Swidsinski *et al.*, 2005). Additionally, the murine small intestine is nearly bacteria-free in contrast to a high bacterial density in the colon (Swidsinski *et al.*, 2005). Thus, the use of small intestine for primary tissue culture enabled a specific bacteria-cell interaction in contrast to the colon. In order to maintain an intact cell organization and to prevent any stress that might influence the cellular gene expression profile, small tissue pieces were used.

Realtime RT-PCR of mRNA from these small intestine tissue pieces after coculture with EcN revealed a 13-fold increase of MCP-1 expression compared to non-treated tissue (fig. 23).

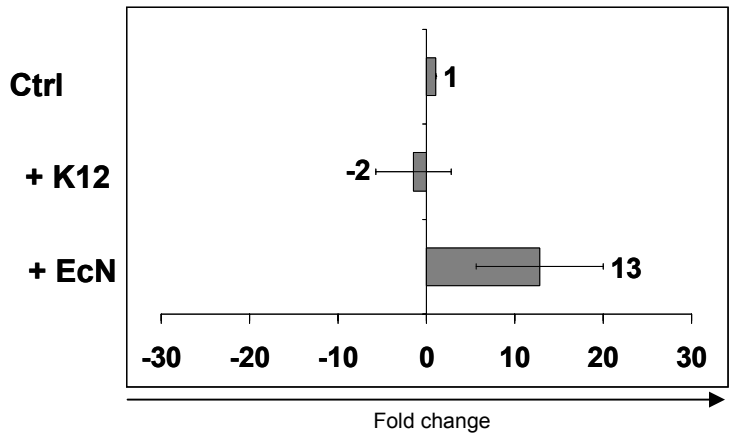

Figure 23. MCP-1 gene expression in small intestine. After smooth removal of the mucus layer, 3-5 tissue pieces from small intestine were cocultured with *E. coli* MG1655 (+ K12) or EcN (+ EcN). Relative mRNA amounts were normalized with respect to expression levels of tissue pieces cultured in cell culture media without antibiotics (fold change = 1). Data are presented as mean of two independent experiments.

In contrast to EcN, coculture with *E. coli* MG1655 yielded an MCP-1 down-regulation, confirming the results obtained from experiments with confluent human intestinal epithelial cell lines.

Summarized, these data obtained from primary mouse tissue culture confirmed the data of cell culture experiments with two human intestinal epithelial cell lines and the finding that EcN is able to specifically induce a proinflammatory host response *in vitro*.

4. Summary

Beneficial effects of probiotics have been demonstrated by using them as therapeutic alternatives for the treatment of human IBDs as well as with *in vitro* and *in vivo* experiments. Several mechanisms of action of probiotics are proposed, but the molecular mechanisms underlying the probiotic effect still remain to be elucidated.

Whole genome expression analyses revealed 126 genes specifically regulated after treatment of confluent Caco-2 cells with EcN. The comparison of data obtained from array data analysis from non-treated Caco-2 cells and those, cocultured with EcN for 6 hours, revealed among others an up to 10-fold increased expression of genes encoding the proinflammatory molecules MCP-1, MIP-2α and MIP-2β. In addition, high amounts of MCP-1 protein were secreted after EcN treatment. Elevated mRNA levels of MCP-1, MIP-2α and MIP-2β could be confirmed by a further human intestinal epithelial cell line. Furthermore, *ex vivo* experiments also revealed an EcN specific up-regulation of MCP-1 in mouse IECs. The EcN specific temporary change of MCP-1 gene expression could neither be induced with bacteria CM nor with paraformaldehyde inactivated organisms suggesting that for EcN mediated MCP-1 gene expression viable bacteria are necessary.

Thus, EcN initiates a proinflammatory response of human and mouse IECs mainly demonstrated by the temporary up-regulation of MCP-1 gene and protein expression.

II. Results Part II

5. Background

The epithelium of the gastrointestinal tract serves as one of human's primary interfaces with the outside world. Apart from digestion and effective absorption of nutrients and electrolytes, the regulation of the transport of macromolecules through the epithelial barrier is an additional fundamental function of the gastrointestinal tract. The mucosal surface of the intestinal epithelium is in constant contact with abundant populations of microbes and their metabolites. The physical barrier formed by the epithelial cells and intercellular junctions exclude the majority of these microbes and their metabolites (i.e. toxins and immuno-stimulatory compounds) from internal access to the subepithelial cells. Disruption of the integrity of the intestinal epithelial barrier alters paracellular permeability and is a key feature of IBDs. The role of a 'leaky gut' in the pathogenesis of gastrointestinal diseases is of increased interest and the use of probiotics as potential therapeutic agents in gastrointestinal diseases is promising. Several convincing clinical studies have proven the beneficial effect of probiotics for remission maintenance of pouchitis, CD and UC (Guslandi *et al.*, 2000; Gionchetti *et al.*, 2003; Kruis *et al.*, 2004).

Apart from modulation of the intestinal immune system and from inhibition of pathogenic bacteria, modulation of the epithelial and mucosal barrier function is one of the potential mechanisms of action of probiotics (Sartor *et al.*, 2004; Dotan and Rachmilewitz, 2005). However, the exact mechanisms by which probiotics can influence the intestinal barrier function remain unclear. Adherence to mucosal surfaces, inhibition of attachment of pathogenic bacteria and enhanced mucin production may be properties in improving mucosal barrier function (Mack *et al.*, 1999; Mack *et al.*, 2003; Resta-Lenert and Barrett, 2003). Studies with trinitrobenzene sulphonic acid induced colitis in rats have shown that colonization with *Lactobacillus casei* ameliorates mucosal injury and prevents barrier disruption (Llopis *et al.*, 2005). *Streptococcus thermophilus* and *Lactobacillus acidophilus* can prevent invasion of enteroinvasive *E. coli* (EIEC) and do enhance intestinal epithelial barrier function by amplifying phosphorylation of the TJ proteins occludin and ZO-1 (Resta-Lenert and Barrett, 2003).

6. Aims of the study

The proposed influence of probiotics on the integrity of the intestinal epithelial barrier gave reason to investigate the impact of probiotic EcN on expression of ZO-1, a key molecule of the tight junctional complex. For this purpose, gnotobiotic mice were colonized with EcN, their IECs were isolated and analyzed further. The detailed examination included:

- Establishment of a bacterial colonization protocol for gnotobiotic mice
- Preparation of murine intestine and isolation of IECs
- RNA purification from isolated IECs and cDNA synthesis
- Determination of ZO-1 mRNA and protein expression
- Analysis of ZO-1 mRNA and protein expression in human IECs
- Transfection of human IECs with a ZO-1/GFP fusion protein
- Investigation of the impact of ZO-1 overexpression on epithelial barrier function

7. Results

7.1 Bacterial colonization of gnotobiotic mice

Rearing animals under germ-free conditions has developed into a scientific field of its own, termed gnotobiology from the Greek words gnosis (= knowledge) and bios (= life). The power of germ-free technology lies in the ability to control the composition of the environment and provides important information about how single bacteria influence the gene expression of the host.

Considering these facts, a model for colonization of gnotobiotic mice with EcN and *E. coli* MG1655 was established in collaboration with the Institute for Laboratory Animal Science and Central Animal Facility at the Hannover Medical School. Mice bred under germ-free conditions were taken from isolators and placed into gnotocages, which are small commercially available containers (Nunc, Wiesbaden, Germany) with custom-made filter caps. The cages are able to maintain sterile experimental conditions for up to 7 days. Three mice per group were inoculated under a clean bench by oral gavage with $1x10^9$ CFU of the respective bacterial strain (fig. 24). In order to ensure a stable colonization of the animals, inoculation was repeated after 3 days.

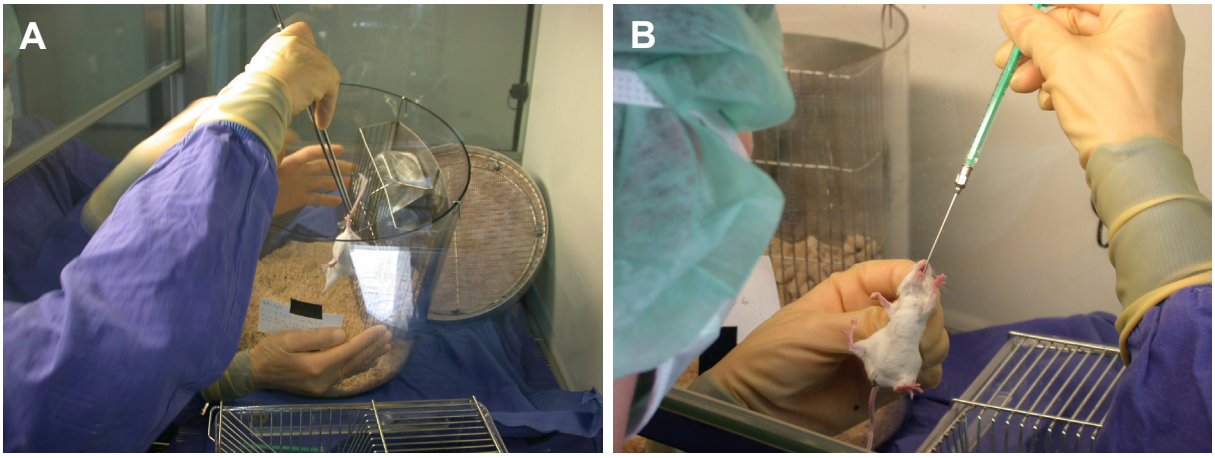

Figure 24. Application of bacteria to gnotobiotic mice.
Gnotocages with gnotobiotic mice were arranged under a clean bench (A) and bacteria were given by oral gavage with a syringe and a blunt ended needle (B).

After 6 days of colonization, fecal CFU were determined by dissolving mouse feces in LB media to a final concentration of 300 mg/ml and plating 10-fold dilution series onto LB agar plates for colony count.

As depicted in table 7 gnotobiotic mice were colonized with similar numbers of EcN and *E. coli* MG1655 in both experiments. Feces of mice that received PBS remained sterile, thus demonstrating the functionality of the gnotocage system.

Table 7. CFU grown from feces of gnotobiotic mice colonized with *E. coli*.

Bacterial strain	CFU/g feces	
	Experiment 1	Experiment 2
EcN	$6.6x10^9$	$4.9x10^9$
E. coli MG1655	$5.9x10^9$	$5.6x10^9$

Upon necropsy of mice that had received either EcN or PBS no macroscopical differences of the animal intestines could be observed (fig. 25).

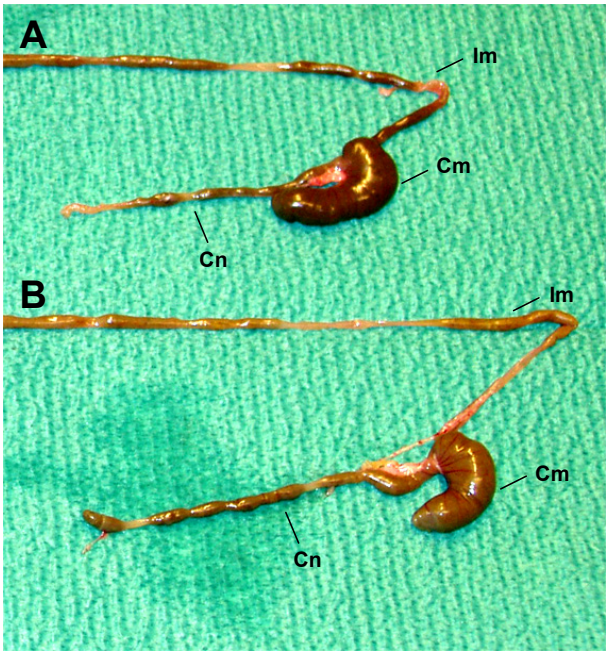

Figure 25. Macroscopic presentation of mice intestines.
Intestine of a gnotobiotic mouse that received sterile PBS (A) and intestine of a gnotobiotic mouse that was colonized with EcN (B). Im indicates ileum; Cm, cecum and Cn, colon.

7.2 Isolation of murine IECs

IECs are uniquely positioned to serve as direct link between the host immune system and the external environment. In their normal state, mucosal surfaces of the alimentary tract are exposed on the luminal surface to high concentrations of foreign antigens, while at the same time, they are intimately associated with the immune system via the subepithelial lymphoid tissue. Consequently, the epithelium together with the intercellular junctions - mainly TJs - forms an important physical barrier. TJs have become increasingly recognized for their role in disease states and microbial infections, since disruption of the intestinal barrier is an essential primary mechanism for most of the pathogenic bacteria to further invade the host cells. Since EcN is in wide use as therapeutic agent in intestinal disorders and probiotics like lactobacilli have been shown to prevent barrier disruption by enteropathogenic bacteria, the impact of probiotic EcN on ZO-1 mRNA and protein expression was investigated.

Therefore, IECs from small intestine of colonized gnotobiotic mice were isolated and sorted by a fluorescence-activated cell scan (FACS) sorting protocol. Staining with anti-CD45 APC antibody resulted in depletion of hematopoetic cell types from the entire epithelial cells. Using a MoFlow cell corter (Cytomation, Fort Collins, CO, USA) the remaining epithelial cell populations could be distinguished by cell granularity and size. This sorting analysis revealed that more than 80% of the isolated intestinal cells are IECs (fig. 26). Re-analysis of the sorted cells demonstrated an IEC purity of greater than 93% (fig. 26).

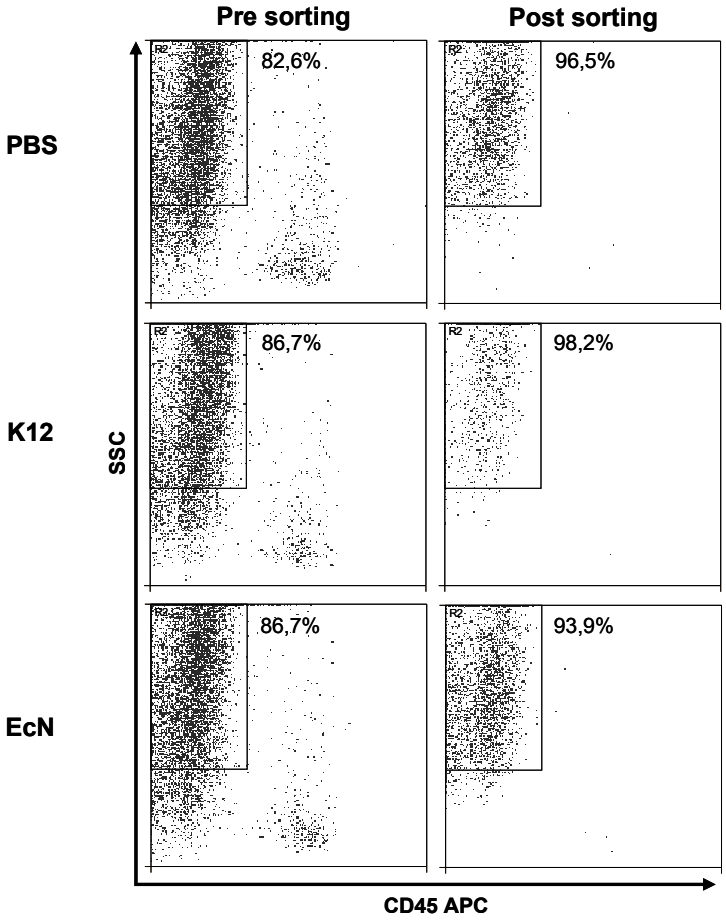

Figure 26. Isolation of IECs by FACS sorting.
Whole intestinal cell populations from gnotobiotic mice, treated either with PBS, *E. coli* MG1655 (K12) or EcN, were labelled with anti-CD45 APC antibody. IECs were identified by granularity and size. Additionally, CD45 positive cells were excluded.

7.3 Realtime RT-PCR revealed increased ZO-1 gene expression in gnotobiotic mice colonized with EcN

For further expression analysis, mRNA of $1x10^6$ sorted IECs was isolated and reversely transcribed. To prove whether cDNA synthesis was successful, RT-PCR for the housekeeping gene ribosomal protein 9 (RPS9) was performed (fig. 27 A).

Subsequently, quantification of ZO-1 gene expression changes in response to EcN were determined by realtime RT-PCR. As depicted in figure 27 B colonization of gnotobiotic mice with probiotic EcN resulted in a 4-fold up-regulation of ZO-1 mRNA expression compared to only 0.3-fold in mice colonized with *E. coli* MG1655.

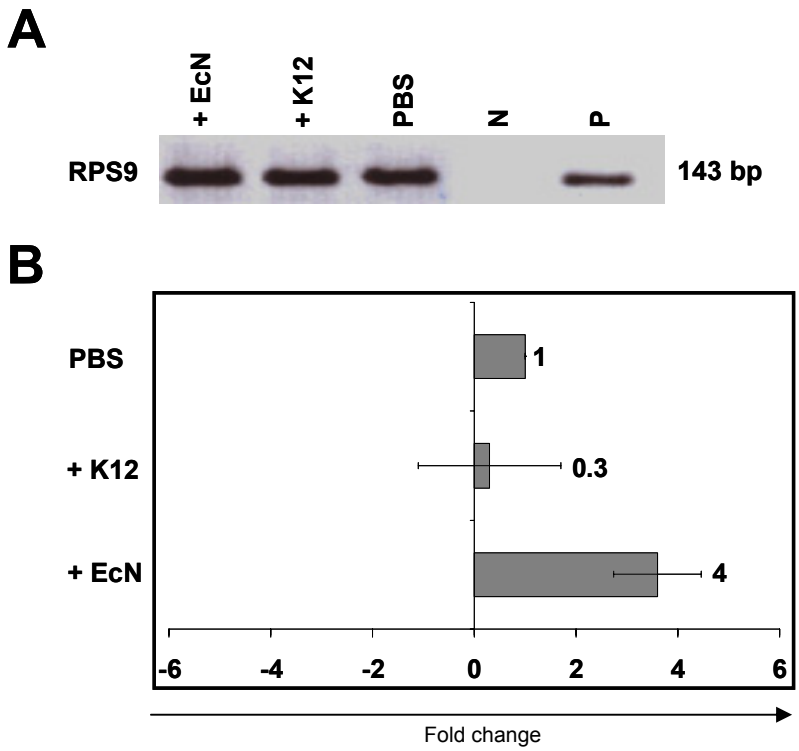

Figure 27. ZO-1 mRNA expression in IECs from gnotobiotic mice.
Gnotobiotic BALB/c mice were colonized with *E. coli* MG1655 (+ K12) or EcN (+ EcN) for 6 days, respectively. (A) RPS9 mRNA expression levels were determined by RT-PCR. N indicates negative control; P, cDNA positive control. (B) Quantitative ZO-1 mRNA expression levels. Application of PBS served as negative control. Relative mRNA amounts were normalized with respect to expression levels of IECs of mice who received PBS (fold change = 1). Data are presented as mean of three independent experiments (n = 3/group).

7.4 ZO-1 protein expression in small intestine

The observation of increased ZO-1 expression at mRNA level, provoked the question, whether elevated amounts of ZO-1 protein could also be detected. Therefore, sections of small intestine from gnotobiotic control mice and animals colonized with EcN were stained immunohistochemically.

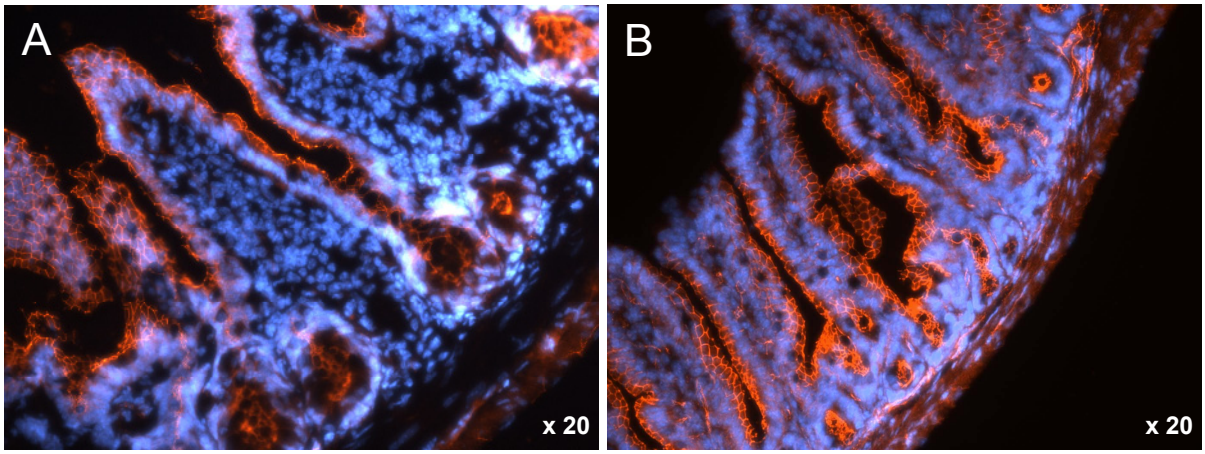

Figure 28. ZO-1 distribution in small intestine.
Immunohistochemical staining of tissue sections of small intestine from gnotobiotic control mice (A) and mice colonized with EcN (B) for 6 days with a fluorescent anti-ZO-1 antibody (orange).

Figure 28 visualizes the ZO-1 distribution along the surface of the crypts. Comparison of ZO-1 staining in sections of small intestine of control mice (fig. 28 A) revealed no detectable differences to those colonized with EcN (fig. 28 B). This is most likely due to the very intense basic fluorescence of the anti–ZO-1 antibody. Therefore a more sensitive method for analysis of ZO-1 protein expression was performed (fig. 29).

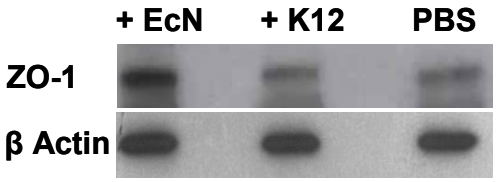

Figure 29. ZO-1 protein expression in IECs of gnotobiotic mice colonized with EcN or *E. coli* MG1655.
Western blot analysis of small IECs of gnotobiotic mice colonized with EcN (+ EcN) or *E. coli* MG1655 (+ K12) and of control mice (PBS) was done either with anti-ZO-1 or anti-β Actin antibody.

$2x10^5$ sorted IECs per group were used for Western blot analysis with anti-ZO-1 or anti-β-Actin antibody, a protein ubiquitously expressed in cells. Although no differences could be detected by immunohistochemical staining, Western blot analysis revealed that ZO-1 protein amounts of mice treated with EcN were much higher compared to those that had received *E. coli* MG1655 or PBS.

These data clearly demonstrate that EcN specifically up-regulates ZO-1 expression at mRNA as well as at protein level. These *in vivo* findings suggest that probiotic EcN may improve the epithelial barrier function through up-regulation of ZO-1.

7.5 Up-regulation of ZO-1 gene expression in murine primary intestinal tissue

Investigation of the influence of EcN on *in vivo* ZO-1 gene expression in gnotobiotic mice precipitated the question, if this observation could be confirmed with primary mouse epithelial cells from conventionally reared animals. Preparation of intestinal tissue pieces was performed according to published protocols with slight modifications (Rogler *et al.*, 1998; Grossmann *et al.*, 2003; Cima *et al.*, 2004). Freshly isolated tissue pieces of murine small intestine (3-5 pieces per well) were cocultured with $1x10^6$ CFU EcN or *E. coli* MG1655, respectively.

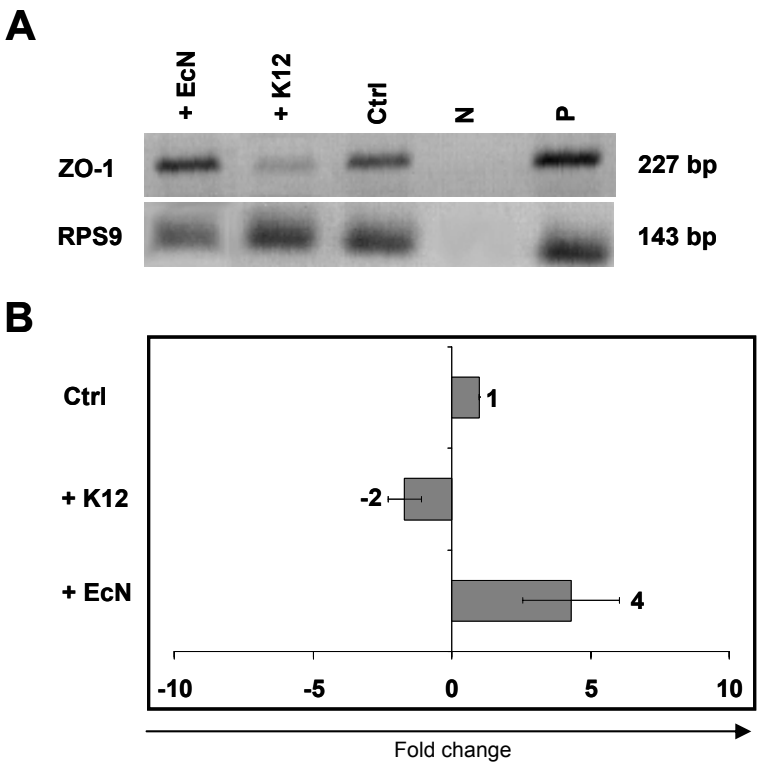

Figure 30. ZO-1 mRNA expression in small intestine of conventional mice.
(A) Tissue pieces from small intestine of BALB/c mice were cocultured with EcN (+ EcN) or *E. coli* MG1655 (+ K12) and mRNA expression levels were determined by RT-PCR. N indicates negative control and P, cDNA positive control. (B) Quantitative expression analysis was performed by realtime RT-PCR. Relative amounts of mRNA were normalized with respect to expression levels of tissue pieces cultured in cell culture media without antibiotics (fold change = 1). Data are presented as mean of three independent experiments (n = 3).

Higher bacterial counts resulted in rapid acidification of cell culture media due to resident murine microflora on the tissue pieces. After 6 hours of coculture, mRNA from the tissue pieces was isolated, reversely transcribed and RT-PCR for RPS9 and ZO-1 was performed. As depicted in figure 30 A, cDNA synthesis was successful. Furthermore, EcN specific up-regulation of ZO-1 mRNA expression was already detectable at this level.

Quantitative realtime RT-PCR even revealed that *E. coli* MG1655 caused a 2-fold down-regulation of ZO-1 gene expression. By contrast, ZO-1 mRNA levels were 4-fold increased after EcN treatment compared to non-treated tissue pieces (fig. 30 B). Thus, the *in vivo* observation of an EcN specific of ZO-1 gene expression in gnotobiotic mice can be confirmed *ex vivo* with conventional animals.

7.6 EcN affects up-regulation of ZO-1 gene expression in human IECs

Based on the animal data, now the question was addressed whether EcN could also trigger ZO-1 expression in human IECs. For that reason, further experiments were performed using Lovo cells. In the following, confluent Lovo cells were cocultured with EcN or *E. coli* MG1655 with an MOI of 1 for 6 hours.

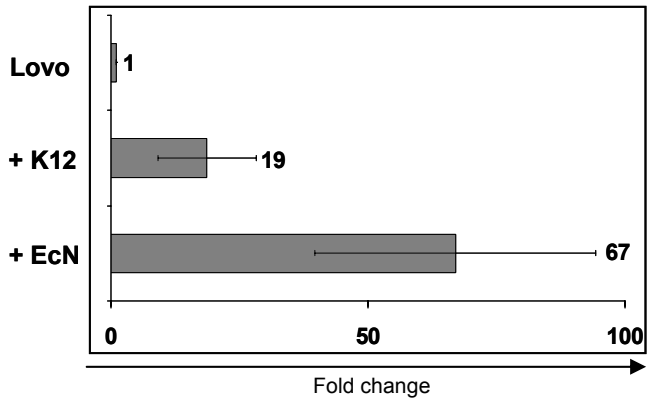

Figure 31. ZO-1 gene expression in human IECs.
Relative mRNA expression levels of ZO-1 in confluent Lovo cells cocultured with EcN (+ EcN) or *E. coli* MG1655 (+ K12) for 6 hours, respectively. Untreated Lovo cells served as control. Quantification of mRNA expression levels were determined by realtime RT-PCR. Relative mRNA amounts were normalized with respect to expression levels of untreated cells (fold change = 1). Data are presented as mean of three independent experiments.

Quantification of the mRNA levels revealed significant ZO-1 up-regulation also *in vitro* (fig. 31). In addition, the relative amount of mRNA in EcN treated cells was about 4-fold higher than in cells cocultured with the control strain *E. coli* MG1655.

To discover potential changes at protein level mediated through EcN treatment, Western blot analysis was performed with anti-ZO-1 and anti-β Actin antibody using $2x10^5$ Lovo cells either untreated or cocultured with EcN or *E. coli* MG1655 (fig. 32).

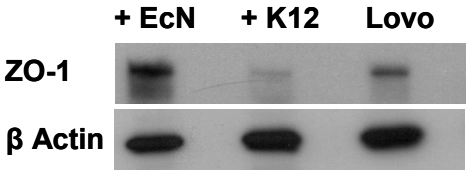

Figure 32. Detection of ZO-1 protein in Lovo cells.
Confluent Lovo cells cocultured with EcN (+ EcN) or *E. coli* MG1655 (+ K12) for 6 hours, respectively. Cells were homogenized and Western blot analysis was performed with anti-ZO-1 or anti-β Actin antibody. Untreated Lovo cells served as a positive control.

Whereas the level of ZO-1 protein in Lovo cells cocultured with *E. coli* MG1655 was rather low, the coculture with EcN led not only to up-regulation of ZO-1 mRNA levels but also to significant elevation of ZO-1 protein.

These data convincingly demonstrate an EcN specific up-regulation of the TJ protein at mRNA and protein level in human IECs, confirming the mouse *in vivo* data.

7.7 Dependency of increased ZO-1 gene expression on the presence of EcN

In order to investigate whether the EcN mediated up-regulation of ZO-1 expression at mRNA and protein level is dependent on the presence of the probiotic itself, coculture experiments were carried out with confluent Lovo cells over 6 hours. After removal of the bacteria containing supernatant and several washing steps, cells were cultured for an additional 12 hours in cell culture media containing antibiotics.

Compared to the 67-fold up-regulation of ZO-1 mRNA expression as a consequence of EcN treatment for 6 hours (fig. 31), after removal of EcN ZO-1 expression decreased at mRNA (fig. 33 A) as well as at protein level (fig. 33 B).

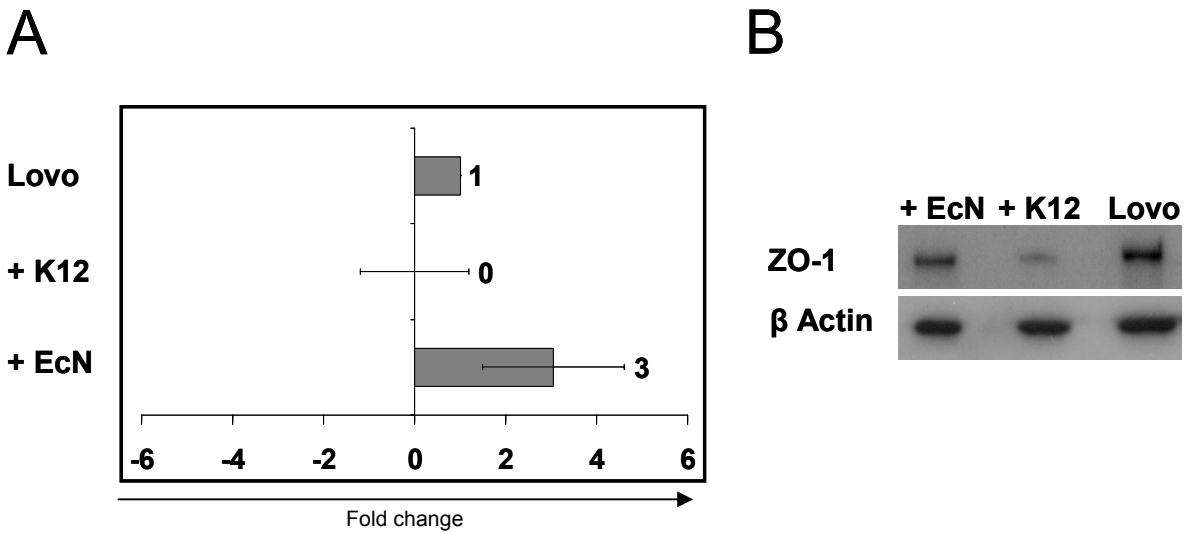

Figure 33. ZO-1 expression in Lovo cells pre-cocultured with EcN.
(A) Relative mRNA expression levels of ZO-1 in confluent Lovo cells cocultured with EcN (+ EcN) or *E. coli* MG1655 (+ K12) for 6 hours, respectively. The supernatant was removed and the cells were cultured for an additional 12 hours in cell culture media containing antibiotics. Untreated Lovo cells served as control. Quantitative ZO-1 mRNA expression was determined by realtime RT-PCR. Relative mRNA amounts were normalized with respect to expression levels of untreated cells (fold change = 1). Data are presented as mean of two independent experiments. (B) Western blot analysis of ZO-1 and β Actin expression in Lovo cells after coculture with EcN (+ EcN) or *E. coli* MG1655 (+ K12) for 6 hours, respectively.

However, the ZO-1 mRNA and protein levels were still higher in cells cocultured with EcN compared to those treated with *E. coli* MG1655. Nevertheless, these results suggest that the presence of EcN is essential for a significantly elevated ZO-1 expression.

7.8 Overexpression of ZO-1 protein reduces invasion of enteropathogenic bacteria

Previous studies have reported that EcN protects gnotobiotic piglets from an infection by *Salmonella* spp. (Mandel *et al.*, 1995). Furthermore, EcN significantly reduces the invasion of *S. typhimurium*, *Yersinia enterocolitica*, *S. flexneri*, *Legionella pneumophila* and even of *Listeria monocytogenes in vitro* (Altenhoefer *et al.*, 2004). Because an important virulence factor of pathogenic strains is the ability to disrupt host epithelial barrier function and thereby to invade host epithelial cells, the question had to be addressed whether up-regulation of ZO-1 might interfere with invasiveness of enteropathogens.

To investigate the functional role of ZO-1, Lovo cells were stably transfected with a ZO-1/GFP fusion protein construct kindly provided by Heidi Wunderli-Allenspach (Department of Applied Bioscience, Swiss Federal Institute of Technology, Zürich, Switzerland). Riesen *et al.* have shown that this fusion protein behaves like endogenous ZO-1 protein (Riesen *et al.*, 2002). Another advantage of this construct is the ability not only to overexpress ZO-1 in cells but also to make these cells detectable by fluorescence microscopy or FACS due to coexpression of the green fluorescent protein (GFP) with ZO-1.

Since the ZO-1/GFP construct is very large with a size of almost 10 kb, it was difficult to obtain very high transfection rates. Therefore, it was necessary to concentrate ZO-1/GFP positive cells by sorting and enriching them with a MoFlow cell sorter (Cytomation) (fig. 34).

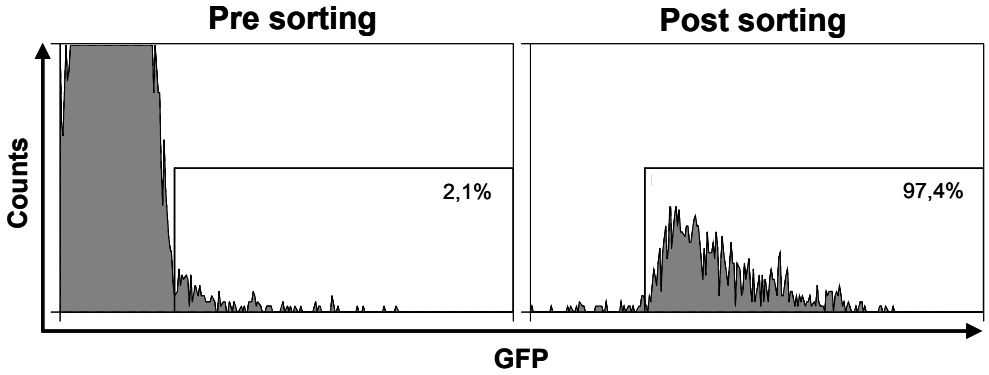

Figure 34. Isolation of ZO-1/GFP transfected cells by FACS sorting.
ZO-1/GFP positive Lovo cells expanded over 3 weeks under selective conditions were sorted based on GFP intensity (Pre sorting). Re-analysis of sorted cells revealed a high purity of ZO-1/GFP positive cells (Post sorting).

To further evaluate the overexpression of ZO-1 protein, $2x10^5$ non-transfected as well as ZO-1/GFP transfected Lovo cells were homogenized and analysed by Western blot using anti-ZO-1 and anti-β Actin antibody. As shown in figure 35, the ZO-1 protein expression level in ZO-1/GFP transfected Lovo cells is strongly increased. Thus, results from Western blot analysis together with the results obtained from FACS analysis document the successful transfection.

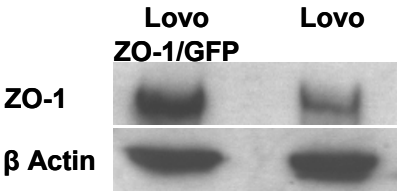

Figure 35. Western blot analysis of ZO-1/GFP transfected Lovo cells.
ZO-1/GFP transfected Lovo cells were homogenized and Western blot analysis was performed using anti-ZO-1 and anti-β Actin antibody. Non-transfected cells served as control.

ZO-1/GFP transfected cells were grown 4 days until confluence and cocultured with EHEC126814, EIEC 4608-58 and *S. typhimurium* aroA for 6 hours, respectively, using an MOI of 1. Following this infection period, extracellular bacteria were killed by antibiotic treatment and intracellular bacteria were enumerated by plate count subsequent to Triton X-100 lysis of the cells.

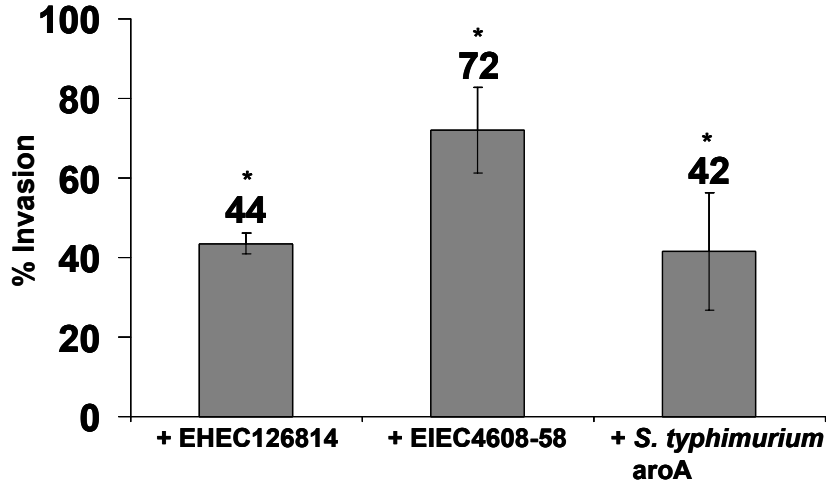

Figure 36. Reduced invasion of enteropathogens in ZO-1/GFP transfected cells.
ZO-1/GFP transfected and non-transfected Lovo cells were cocultured with EHEC126814 (+ EHEC126814), enteroinvasive *E. coli* strain 4608-58 (+ EIEC4608-58) and *S. typhimurium* aroA for 6 hours, respectively. Extracellular bacteria were killed by antibiotic treatment with gentamicin, penicillin and streptomycin. After cell lysis with TritonX-100, intracellular bacteria were enumerated by plate count. The invasion efficiency is expressed as the percentage of the inoculum surviving the antibiotic treatment. The invasion rate of non-transfected cells was set as 100%. Data are presented as mean of three independent experiments (student's t-test: *$p < 0.01$ versus non-transfected cells).

Results of these experiments clearly demonstrated an inhibitory effect of ZO-1 overexpression on the invasiveness of enteropathogenic bacteria. The invasion efficiency was significantly reduced by more than 50% for EHEC126814 and *S. typhimurium* aroA and by 28% for EIEC4608-58 compared to non-transfected cells (fig. 36).

These data strongly suggest that EcN improves the intestinal epithelial barrier function through up-regulation of ZO-1 mRNA and protein expression.

8. Summary

The barrier function of the intestinal epithelium plays a pivotal role as the first line of defense of the host cell. The specialized structure of the epithelium, composed of microvilli of the brush border and TJs, is responsible for a tight barrier. Disturbance of this barrier by pathogenic microorganisms and their toxins may be the initial event for numerous chronic diseases and inflammatory processes. Alteration of TJ structure in UC for instance results in impaired barrier function (Schmitz *et al.*, 1999) Hence, integrity of the intestinal epithelial barrier is of immense importance. This could justify the use of probiotics since one of their potential mechanisms of action is reinforcement of the epithelial barrier function (Gionchetti *et al.*, 2005).

ZO-1 is the key molecule of the TJ complex, linked to the actin filaments and thereby involved in the regulation of paracellular permeability. Colonization of gnotobiotic mice with EcN for 6 days revealed a specific up-regulation of this molecule both at mRNA and protein level in IECs. Additionally, these results could be confirmed with primary IECs of conventional mice. To answer the question whether data obtained from murine IECs could be transferred to the human system, further *in vitro* investigations were performed using the human intestinal epithelial cell line Lovo. Coculture of these cells with EcN for 6 hours also resulted in an EcN specific increase of ZO-1 gene and protein expression. This probiotic mediated effect seems to depend on the presence of viable bacteria since the expression decreased after displacement of EcN. Overexpression of ZO-1 in IECs also significantly reduced the invasion efficiency of enteropathogenic bacteria.

The presented data document a positive influence of the probiotic EcN on ZO-1 expression in mouse and human IECs. This observation implies an important mechanism whereby EcN might protect the host intestinal epithelium against infections with pathogens.

CHAPTER III

Discussion

III. Discussion

The probiotic EcN has been in wide use in the preantibiotic era to treat various diseases of the digestive tract of humans (Nissle A., 1919). However, when the first antibacterial chemotherapeutics had been discovered, knowledge about this strain fell into oblivion. Promising results from several controlled clinical studies, which have proven a role for probiotic therapy in different states of IBDs (Rembacken *et al.*, 1999; Kruis *et al.*, 2004), have recently reawakened substantial interest in EcN. Nonwithstanding the convincing outcome of these clinical trials and even though the strain is obviously a safe carrier for targeted delivery of therapeutical agents (Westendorf *et al.*, 2005), not much is known about the molecular mechanisms responsible for the beneficial effects of EcN application, especially at host level.

Contact between a host and a specific microorganism alters bacterial and eukaryotic gene regulation. The commercial availability of human gene expression analysis arrays offers the possibility to monitor global changes in host gene transcription in response to microorganisms or other stimuli. Thus, to investigate the wide range of intestinal molecular functions that are shaped by probiotic EcN, microarray technology was used for *in vitro* characterization of IEC transcriptional responses evoked by EcN.

As a result of EcN exposure a set of 126 genes specifically regulated in human IECs was identified (fig. 13). Surprisingly, the majority of these genes was also regulated in response to EHEC126814 (fig. 15 C). EHEC is known for more than 2 decades to cause bloody diarrhea as well as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) especially among children between 1 and 6 years of age. Shiga toxins (Stxs) are considered as the major virulence factors of EHEC and are of key importance for the development of HUS (Paton and Paton, 1998). It is generally believed that after intestinal infection with EHEC, Stxs cross the intestinal barrier and bind to host cells. At this point they presumably injure them by inhibition of protein synthesis, stimulation of prothrombotic messages or induction of apoptosis. HUS is the net effect of a variety of interacting factors, including background risk of acquisition, virulence characteristics of the infecting EHEC strain, and exogenous factors. Furthermore, EHEC virulence is due to the expression of a number of adhesins, which allow the initial binding to the host mucosal surface, and effector proteins of the type III secretion apparatus, that interact with host cell components

and signaling pathways, altering their function and thus triggering the formation of the characteristic attaching and effacing phenotype (Roe *et al.*, 2003). In contrast, non-pathogenic EcN lacks all of such virulence factors in the traditional sense. Thus, gene and/or protein up-regulation of the proinflammatory molecules MCP-1, MIP-2α and MIP-2β in human IECs in response to coculture with probiotic EcN (fig. 16a, 18, 19 and 21) was a completely unexpected observation. The genome content of EcN may give explanations for the proinflammatory properties of this strain. Recently, Sun *et al.* (Sun *et al.*, 2005) and Grozdanov *et al*. (Grozdanov *et al*., 2004) have compared the genome of probiotic EcN and the uropathogenic *E. coli* strain CFT073. Interestingly, these genome comparisons revealed a high degree of homology between EcN and *E. coli* CFT073. The existence of about 130 “common virulence factors” in probiotic EcN, as defined by the PRINTS protein fingerprint database, suggests that these proteins cannot be considered as virulence factors per se, but may also contribute to the general fitness of EcN (Sun *et al*., 2005). However, these data also indicate that EcN might be considered a “pathogen to happen”.

MCP-1 is a CC chemokine, produced by many cells, including epithelial cells, that attracts monocytes, memory T lymphocytes and NK cells *in vitro* (Matsushima *et al.*, 1989; Yoshimura *et al.*, 1989; Allavena *et al.*, 1994; Carr *et al.*, 1994). It has been shown that its expression is promoted after exposure to inflammatory stimuli such as interleukin-1 (IL-1) (Reinecker *et al.*, 1995), tumor necrosis factor alpha (TNF-α) or interleukin-4 (IL-4) (Van Coillie *et al.*, 1999). This chemokine is expressed under many pathological conditions, including IBDs. It is suggestive that MCP-1 could play a role in the pathogenesis of mucosal inflammation by influencing the increased migration of monocytes/macrophages, granulocytes, and lymphocytes from the blood stream through the endothelium into both, the mucosa and submucosa during chronic IBDs (MacDermott, 1999). In addition, up-regulated chemokine expression correlates with increased activity of the disease (Banks *et al.*, 2003). Several models of transgenic expression in mice have validated MCP-1 as a predominantly monocytic chemoattractant *in vivo* (Fuentes *et al.*, 1995; Rutledge *et al.*, 1995; Gunn *et al.*, 1997). However, several lines of evidence indicate that MCP-1 might also influence T cell immunity. T lymphocytes express the MCP-1 receptor CCR2, and MCP-1 is a potent chemoattractant for memory T cells *in vitro* (Loetscher *et al.*, 1996). Additionally, MCP-1 expression is associated with the development of

polarized T_H2 responses, since MCP-1 deficient mice are unable to mount T_H2 responses (Lu *et al.*, 1998; Gu *et al.*, 2000). Thus, MCP-1 influences both, innate immunity through effects on monocytes, and adaptive immunity through control of T_H polarization.

The data presented in this study demonstrate for the first time, that probiotic EcN up-regulates MCP-1 gene and protein expression in human and mouse IECs. Up-regulation of this proinflammatory molecule in IECs, as the first line of contact between the bacteria and the host, was to date only observed in context with lactobacilli. Recently published data by Lan *et al.* have shown that exposure of primary murine colonic epithelial cells to *Lactobacillus rhamnosus* GG resulted in an exclusive induction of MCP-1 expression (Lan *et al.*, 2005). Other authors have described this phenomenon only after direct contact between probiotic microorganisms and immune cells, like macrophages and dendritic cells. Probiotic *Lactobacillus rhamnosus* GG infection of human macrophages, which have a central role in the innate immune response to bacteria, revealed induction of MCP-1 expression and T_H1 cell migration (Veckman *et al.*, 2003). Regarding EcN intersting data were presented recently by Sturm *et al.* The authors could show, that CM of this probiotic inhibits peripheral blood T cell cycling and increases the proportion of γ/δ T cells (Sturm *et al.*, 2005).

The neutrophil attracting chemokines MIP-2α and MIP-2β represent two additional molecules involved in inflammatory processes. They exhibit a number of immunoregulatory activities. Interestingly, intraepithelial and lamina propria lymphocytes also show chemotactic activity to MIP-2 suggesting a role for MIP-2 in controlling mucosal lymphocyte migration (Ohtsuka *et al.*, 2001). In addition to MCP-1, MIP-2α and MIP-2β gene expession was also increased in human IECs upon contact with EcN after 6 hours (fig. 16a), reaching peak levels after 24 hours (fig. 20). Other investigations revealed an increase of MIP-2 mRNA only in response to proinflammatory stimuli and inflammation. Subcutaneous injection of MIP-2 into footpads of C3H/HeJ mice elicited an inflammatory response characterized by neutrophil infiltration, suggesting MIP-2 to be an endogenous mediator during inflammatory processes (Wolpe *et al.*, 1988). In addition, MIP-2 has been shown to enhance the inflammatory effects of DSS induced colitis in transgenic mice (Ohtsuka and Sanderson, 2003). Release of this chemokine from IECs is increased by

infection with *Clostridium difficile* (Castagliuolo *et al.*, 1998), the pathogen responsible for antibiotic-associated pseudomembranous colitis (Dillon *et al.*, 1995; Borriello, 1998; Nusrat *et al.*, 2001). Stimulation of IECs with proinflammatory IL-1β also resulted in increased expression of MIP-2 (Ohno *et al.*, 1997), whereas MCP-1 expression was increased after stimulation with IL-4 and IFNγ (Winsor *et al.*, 2000). This increased expression of MIP-2 is amplified by butyrate, a metabolite of nonpathogenic resident bacteria. This is a potential mechanism by which resident bacteria may regulate inflammatory processes in the small intestine (Ohno *et al.*, 1997).

The NF-κB signaling pathway is one of the most important pathways in proinflammatory signaling (Ghosh *et al.*, 1998; Maeda *et al.*, 2005). Activation of the NF-κB pathway can be initiated by a myriad of stimuli, including endogenous mediators (e.g. TNF, IL-1β) and bacteria, their products or components (e.g. LPS, peptidoglycan, flagellin, CpG), that bind to TLRs. In resting cells NF-κB translocation into the nucleus is prevented by the IκB proteins NFκBIA (IκBα), NFκBIB (IκBβ) and NFκBIE (IκBε). But activation of this pathway results in degradation of the IκB proteins, NF-κB translocation to the nucleus and subsequent transcription of proinflammatory genes (fig. 37). In case of infections with enteric pathogens this mainly results in secretion of proinflammatory cytokines like IL-8 (Hobbie *et al.*, 1997; Elewaut *et al.*, 1999; Gewirtz *et al.*, 2000).
As indicated by the data shown, MCP-1 gene up-regulation in response to EcN is specific but temporary (fig. 16a, 18 and 20). This observation correlates with the up-regulation of NFκBIA, which is nearly constant over 48 hours, and the only minimally increased NF-κB1 (p105/p50) mRNA level (fig. 17). It can be assumed that the observed EcN specific MCP-1 expression triggers adaptive and innate immune responses and is later on repressed by inactivated NF-κB.

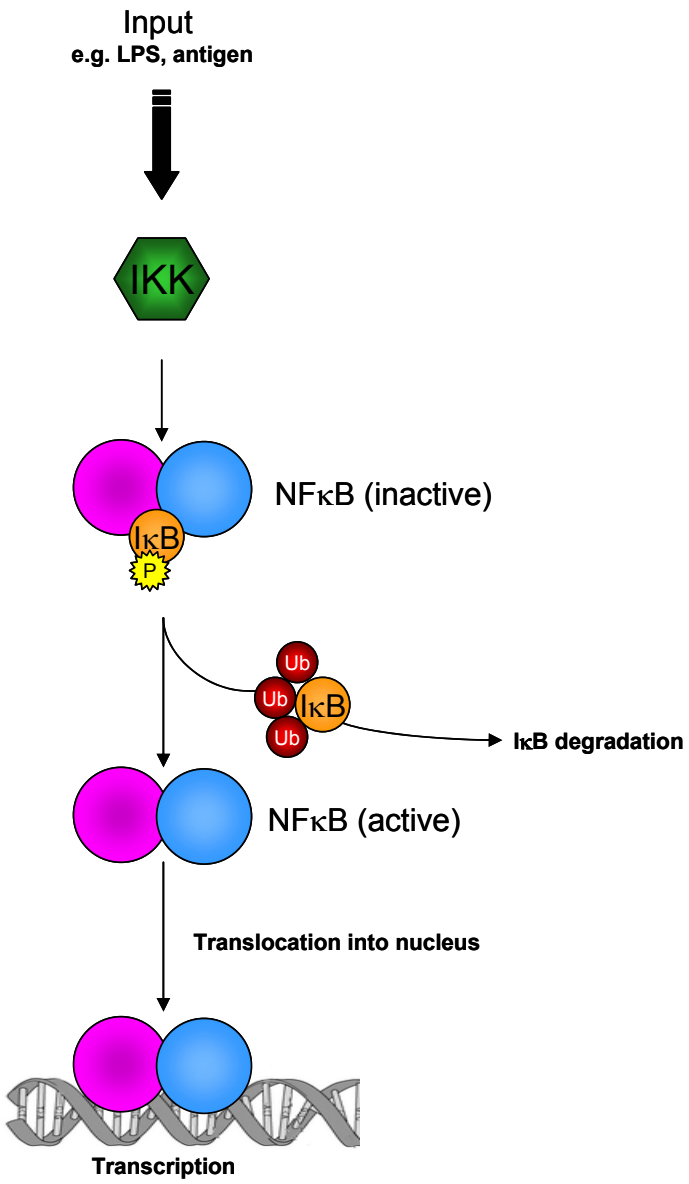

Figure 37. Schematic presentation of the NFkB pathway.
NFκB is composed of two subunits, both homo- and heterodimers, of various members of the Rel family of DNA-binding transcription factors. Several isoforms of NFκB are used by the intestinal epithelium. In resting cells, NFκB is inactive because of association with inhibitor proteins (IκBα, β, ε) that mask the nuclear localization sequence of NFκB, thereby retaining it in the cytoplasm and preventing DNA binding. Various stimuli lead to the activation of NFκB signaling via membrane receptors . Signal-activated phosphorylation of IκBs is mediated by IκB kinases (IKKs). Subsequently, IκBs are ubiquitinylated and finally degraded by the proteasome. This allows nuclear translocation of NFκB and DNA binding, which initiates gene transcription.

Very recently published *in vivo* data obtained from mouse models of systemic *Listeria monocytogenes* infection, demonstrated that *Lactobacillus casei* strain Shirota augments innate immunity in spleen cells by induction of TNF-α, IL-12, IL-18 and IFN-γ production through activation of both NF-κB and p38 MAPK pathways and additionally up-regulates TLR2 and NOD2 mRNA expression (Kim *et al.*, 2006).

Another possibility is the activation of MCP-1 via an NF-κB independent pathway. The JNK/AP-1 signaling cascade is a likely candidate, since transcription and secretion of MCP-1 can also be activated through this pathway triggered by TLR4 signaling (Paik *et al.*, 2003). It is a well-known fact that TLR4 and TLR5 recognize LPS (Hoshino *et al.*, 1999) and bacterial flagellin (Hayashi *et al.*, 2001), respectively.

Both receptors can induce the transcription of MCP-1, MIP-2α and MIP-2β (Schaefer *et al.*, 2004). Induction of DSS colitis in TLR4$^{-/-}$ mice led to an altered neutrophil recruitment due to diminished MIP-2 expression by lamina propria macrophages. Thus, TLR4 participates in the intestinal immune response to luminal bacteria and the development of colitis (Fukata *et al.*, 2005). It has been shown, that commensals such as *E. coli* MG1655 induce NF-κB activation through the TLR5 signaling pathway (Bambou *et al.*, 2004), whereas commensal aflagellated *Bacteroides vulgatus* activates NF-κB through the TLR4 pathway (Haller *et al.*, 2002). Since both, EcN and *E. coli* MG1655 possess flagellin and LPS, it can be speculated that an additional component of EcN not yet identified is able to induce MCP-1 gene expression. However, the semirough O6 LPS phenotype of EcN, which is different from O6 LPS of uropathogenic *E. coli* and which is responsible for serum sensitivity of EcN (Grozdanov *et al.*, 2002), might also play a role in this context.

EcN's ability to induce local proinflammatory immune responses in IECs is a means of this strain modulating host defense mechanisms. A protective immunological barrier will be established by initiation of a mild inflammation.

The definition of the term "probiotic" stresses the importance of the viability of the microbes. With regard to the properties of probiotics, e.g. resistance to pancreatic enzymes, acid, bile and adhesion to the intestinal mucosa to exert their benefits like stimulation of mucosal barrier function, or altering immunoregulation, the viability of probiotics seems to be essential to generate these effects. But in the past years, this hypothesis was challenged.
The beneficial effects of lactic acid bacteria, often used as dietary supplements in so called functional food and representing the predominant fermentative microbes in dairy foods such as yoghurt and cheese (Salminen *et al.*, 1998), can be expected when viable bacteria are able to survive the passage through stomach and digestive system and do colonize the gut (Kailasapathy and Chin, 2000). Comparison of the influence of viable and nonviable lactic acid bacteria on the intestinal mucosal immune system, revealed an enhanced stimulation by viable bacteria. It has been shown, that viable probiotic *Lactobacillus casei* stimulates the intestinal mucosal immune system to a greater extent than nonviable bacteria (Galdeano and Perdignon, 2004). Another study revealed the dependence of the suppressive effect

on EHEC internalization by *Lactobacillus rhamnosus* on the viability of the probiotic (Hirano *et al.*, 2003). Investigations with live probiotic *Streptococcus thermophilus* and *Lactobacillus acidophilus* exposed to cell monolayers limited adhesion, invasion, and physiological dysfunction induced by EIEC, whereas antibiotic killed probiotics were less effective and heat inactivated bacteria had no protective effect (Resta-Lenert and Barrett, 2003). Furthermore, therapeutic effects of EcN in remission maintenance of UC were achieved with viable bacteria given as the drug Mutaflor®. However, it has been suggested that some effects of the probiotics, like induction of heat shock proteins, do not require live bacteria (Petrof *et al.*, 2004).

This is also true for the results obtained in this study. As presented in table 6, induction of EcN specific MCP-1 gene expression is dependent on the viability of the bacteria and is apparently not due to EcN secreted metabolites. In contrast, EcN mediated mRNA expression of MIP-2α and MIP-2β seems to be independent on probiotic viability, as inactivated bacteria induced gene expression as well. Thus, EcN induces a proinflammatory cytokine response which is in part dependent on the viability of the bacteria. The fact that only viable EcN is able to up-regulate MCP-1 gene and protein expression is in line with the finding that the inhibitory effect of EcN on peripheral T cell function depends on live bacteria (Sturm *et al.*, 2005). Regarding infection experiments with enteroinvasive bacteria, only live probiotic *Streptococcus thermophilus*/*Lactobacillus acidophilus* limited pathogenic adhesion and invasion (Resta-Lenert and Barrett, 2003). An important study from Rachmilewitz *et al.* has demonstrated, that for attenuation of experimental colitis live bacteria are not mandatory, since genomic DNA isolated from VSL#3 ameliorated the severity of induced and spontaneous colitis (Rachmilewitz *et al.*, 2004). These results support the provocative concept, that the beneficial effect of probiotics is mediated by recognition of unmethylated CpG DNA sequences, the ligand for TLR9, and live bacteria are not needed. Lammers *et al.* suggested that the genomic DNA of probiotic bacteria has immunomodulatory effects on the host cells (Lammers *et al.*, 2003). DNA preparations from two *Lactobacillus* strains suppressed endotoxin-induced inflammation in a macrophage cell line (Luyer *et al.*, 2005).

Interestingly, recent *in vitro* findings demonstrate EcN mediated activation of the NF-κB promoter in the signaling pathway leading to gene induction of the antimicrobial peptide human beta defensin-2 (hBD-2), secreted by paneth cells, indicating a stimulatory role of probiotic bacteria in the intestinal innate defense

(Wehkamp *et al.*, 2004). Defensins exert their host defense function by killing of microorganisms and regulation of host adaptive immunity against microbial invasion. A decrease or lack of mucosal peptide antibiotics may play a central role in the etiopathogenesis of CD. CD patients with ileal involvement are characterized by a diminished expression of the ileal Paneth cell defensins (Wehkamp *et al.*, 2005).

However, secretion of defensins by intestinal epithelia is only one of the multifaceted host defense mechanisms against pathogens. The barrier function of the intestinal epithelium plays a pivotal role in the prevention of intestinal pathology. Disturbance of this barrier may be the cause of numerous chronic diseases and inflammatory processes. Maintenance of barrier integrity by the use of probiotics is one of the discussed mechanisms of probiotic action (Gionchetti *et al.*, 2005).
Several studies have shown disruption of the TJ barrier of IECs through enteropathogenic *E. coli* strains *in vivo* and *in vitro* (Simonovic *et al.*, 2000; Dean and Kenny, 2004; Muza-Moons *et al.*, 2004; Shifflett *et al.*, 2005). Infection of T84 cell monolayers with *S. flexneri* displayed a *S. flexneri* mediated modulation of the function of TJ components, resulting in the specific temporal removal of claudin-1, the dephosphorylation of occludin and the down-regulation of ZO-1 (Sakaguchi *et al.*, 2002). *S. typhimurium* infections induce alterations of the cytoskeleton and associated rapid increase of TJ permeability (Jepson *et al.*, 1995; Jepson *et al.*, 2000). These studies identify the epithelial barrier as an essential target of enteric pathogens. Consequently, maintenance of an intact epithelial barrier is of major importance for protection of the host against pathogens.

In the past years, various investigations clearly demonstrated the effectiveness of probiotics in preventing invasion and adhesion of enteropathogenic bacteria. Moreover probiotics are able to attenuate increased epithelial permeability resulting from bacterial invasion.
The data presented in this study demonstrate for the first time a direct influence of EcN on expression of TJ associated genes in IECs: probiotic EcN specifically up-regulated ZO-1 both at mRNA and at protein level *in vitro* as well as *in vivo* (fig. 27, 29, 30, 31 and 32). This finding is consistent with recently published data regarding the influence of probiotics by affecting TJ proteins. Pre-exposure with probiotic *Lactobacillus acidophilus* and *Streptococcus thermophilus* evoked maintenance

(actin, ZO-1) or enhancement (actinin, occludin) of cytoskeletal and TJ protein phosphorylation (Resta-Lenert and Barrett, 2003). Moreover, *in vivo* administration of *Lactobacillus acidophilus* improved occludin expression (Qin *et al.*, 2005). In contrast to the data from Qin *et al.*, who infused the probiotic by jejunostomy tube three times per day for 1-5 days, the method used in this study was more physiological, since EcN was applied orally to mice on day 1 and 3 and IECs were isolated after 6 days of colonization. In addition, EcN specific increase of ZO-1 gene and protein expression in Lovo cells was dependent on the presence of the probiotic (fig. 33). This observation is in line with the results of the study from Resta-Lenert and Barrett, where the beneficial effect on the barrier function was observed for probiotic treatment alone and furthermore was dependent on the bacterial viability (Resta-Lenert and Barrett, 2003).

As shown in fig. 36 overexpression of ZO-1 in IECs significantly reduced the invasiveness of enteropathogenic *S. typhimurium* aroA, EHEC and EIEC, corroborating the hypothesis of a reinforced intestinal barrier through EcN mediated ZO-1 up-regulation. The presented data might correspond with previously published results and might give explainations for the observations presented below. Pretreatment with EcN and coinfection with adherent-invasive *E. coli* strains revealed significant inhibitory effects of EcN on adhesion and invasion of the pathogens (Boudeau *et al.*, 2003). Sherman *et al.* have shown that *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* have the ability to attenuate the pathogen-induced decrease in transepithelial resistance (TER) (Sherman *et al.*, 2005). Incubation of IECs with *Salmonella enterica* serovar Dublin alone resulted in a significant decrease of TER, whereas VSL#3 alone increased TER and stabilized it when cocultured with *Salmonella enterica* serovar Dublin (Otte and Podolsky, 2004). Infection experiments with EPEC revealed that *Lactobacillus casei* prevented EPEC-induced increase in paracellular permeability, the inhibition of TER decrease and ZO-1 redistribution in T84 cells (Parassol *et al.*, 2005). Since *Lactobacillus casei* interacts with IECs and decreases TNF-α (Borruel *et al.*, 2003), known to down-regulate the transcription level of occludin (Mankertz *et al.*, 2000), these effects might be dependent on *Lactobacillus casei* induced cytokine production in IECs. TNF-α induced increase in intestinal TJ permeability is associated with NF-κB dependent down-regulation of ZO-1 (Ma *et al.*, 2004). Data published very recently have shown, that the probiotic yeast *S. boulardii* induces a decrease of TNF-α in EHEC-infected T84 cells

(Dalmasso *et al.*, 2006). However, an influence of EcN on regulation of TNF-α in IECs has not been described yet.

Since close adherence of enteric pathogens to host epithelial surfaces is one of the prerequisites for a successful infection, another potential anti-invasion mechanism seems to be blocking of epithelial cell surface receptors. Prevention of adhesion and invasion of pathogens by competition for binding sites has mainly been demonstrated for non-coliform bacteria such as lactobacilli and bifidobacteria (Coconnier *et al.*, 1993; Bernet *et al.*, 1994; Candela *et al.*, 2005). The combined treatment of *Lactobacillus acidophilus* and *Streptococcus thermophilus* also significantly limited adhesion and invasion of EIEC (Resta-Lenert and Barrett, 2003). One study addressing this topic was carried out with EcN. It suggests EcN to interfere with enteropathogenic invasion of IECs not through direct physical contact via bacterial adhesins but rather by secreted components (Altenhoefer *et al.*, 2004). Furthermore, adherence of EPEC to HT29 cells can be inhibited by the induction of mucin expression by certain lactobacilli (Mack *et al.*, 1999).

The present study clearly demonstrates the EcN specific ZO-1 gene and protein up-regulation in IECs *in vitro* and *in vivo*. It can be speculated that EcN thereby enhances barrier integrity, which might provide an important *in vivo* mechanism to protect the host cells against enteropathogens. In addition, an improved mucosal barrier function might be an important aspect in the remission maintenance of human intestinal disorders like IBDs.

This study provide novel insights into the probiotic EcN-host interaction. On the one side the results suggest EcN to establish an immunological barrier and, on the other side, to enhance the barrier function of the intestinal mucosa. Data also emphasize that beneficial effects of probiotics do not only rely on a single mechanism but are rather complex.

Further prospects of this study will include *in vitro* investigations of primary human IECs isolated from intestinal tissue of patients undergoing surgical resection for carcinoma or from endoscopic biopsies. It is planned to compare IECs from healthy

individuals to those from patients with UC or CD with respect to EcN mediated MCP-1 and ZO-1 up-regulation.

Additionally, the cell coculture system described here will serve as a read-out to identify the effector molecules of EcN that are responsible for the regulatory phenomena presented in this study.

Eventually, these experiments will contribute to a better understanding of the beneficial effects of EcN and other probiotics, and will support development of optimized microorganisms for therapeutic use.

CHAPTER IV

Materials and Methods

IV. Materials and Methods

1. Cell culture

The human colon adenocarcinoma cell lines Caco-2 and Lovo were maintained in IMDM cell culture medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany) and 250 µg/ml penicillin/streptomycin (Invitrogen) at 37°C in a cell culture incubator. Caco-2 cells were used from passage 12 – 26. Cells were split twice a week at a ratio of 1:3. 4 – $8x10^5$ cells per well were seeded in six well plates (Nunc) and cultured for approximately four days until confluence.

2. Preparation of microorganisms

EcN was isolated from a tablet of Mutaflor® (Ardeypharm, Herdecke, Germany) and cultured on MacConkey plates (Oxoid, Wesel, Germany). The isolate was serotyped and confirmed by EcN specific PCR. *E. coli* K12 laboratory strain MG1655 was kindly provided by Ulrich Dobrindt (Institute for Molecular Infection Biology, Würzburg, Germany). The EHEC strain 126814 of serotype O26:H11 was originally isolated at the Hannover Medical School from a 4 year old girl with HUS, whereas the EHEC strain 86-24 of serotype O157:H7 is a meat isolate that originated from an outbreak in Walla Walla, WA, USA. *S. typhimurium* aroA was kindly provided by Holger Lößner (Molecular Immunology, GBF, Braunschweig, Germany). The yeast *S. boulardii* was isolated from a capsule of Perenterol® (Celltech Pharma, Essen, Germany).

Before starting the experiments growth curves were generated in the corresponding medium as well as in IMDM medium without supplements.

All bacterial strains were grown overnight in Luria Bertani (LB) medium at 37°C on a shaker, whereas yeasts were incubated for approximately 24 hours in YPD medium at 37°C with shaking.

For coculture experiments overnight cultures were diluted 1:100 in 50 ml prewarmed IMDM medium containing 10% FCS and grown at 37°C. Bacteria and yeasts were harvested in late logarithmic phase, after reaching an OD_{600} = 1.

For plate culturing and plate count, LB agar plates and YPD agar plates were used, respectively. 30 µg/ml streptomycin were added for culturing of *S. typhimurium* aroA.

LB broth: 10 g Bacto Tryptone
5 g Bacto Yeast Extract
10 g NaCl
added up to 1 l with autoclaved dH_2O

YPD broth: 10 g Bacto Yeast Extract
20 g Bacto Peptone Extract
20 g Glucose
added up to 1 l with autoclaved dH_2O

3. Coculture of cell lines

3.1 Coculture over 6 hours

Confluent Caco-2 and Lovo cells (0.4 – $1x10^7$ cells per well) were washed with phosphate buffered saline (PBS) and cocultured with a low bacterial MOI of 1 in IMDM medium (Invitrogen) containing 10% FCS at 37°C in a cell culture incubator for 6 hours. Due to the slower doubling time of the yeasts, an MOI of 10 was used. The MOI at the beginning of the experiments was estimated by growth curves. Effective MOI was determined the following day by plate count. Bacterial coculture experiments were performed with a final MOI between 0.5 and 1.5, the MOI of the yeasts ranged between 8 and 12. Upon designing bacterial coculture experiments, CFU of EcN and *E. coli* MG1655 were determined after 6 hours by plate count. Both strains reached comparable numbers in cell culture media ranging from 5 – $8x10^8$ CFU/ml. Prior to RNA isolation cells were washed twice with PBS.

3.2 Extended coculture of Caco-2 cells

For experiments with confluent Caco-2 cells investigating time-dependency of gene expression, bacteria CM was collected by centrifugation after coincubation for 6 hours. The pellets were redissolved in 3 ml IMDM medium with 10% FCS containing 200 µg/ml gentamicin (Sigma-Aldrich, Taufkirchen, Germany) and 250 µg/ml penicillin/streptomycin (Invitrogen) to inhibit further bacterial growth and acidification of cell culture media. The suspension was then used to incubate Caco-2 cells for another 42 hours. Prior to RNA isolation cells were washed twice with PBS.

3.3 Measurement of EcN dependent ZO-1 expression in Lovo cells

For extended coculture experiments with Lovo cells, confluent cells were washed extensively after 6 hours coculture and grown for an additional 12 hours in IMDM media with 10% FCS containing 200 µg/ml gentamicin (Sigma-Aldrich) and 250 µg/ml penicillin/streptomycin (Invitrogen) at 37°C in a cell culture incubator.

3.4 Cocultivation of Caco-2 cells with inactivated bacterial pellets or bacteria CM

Bacteria were grown overnight as described above. Following experiments were performed according to a protocol published by Menard *et al.* (Menard *et al.*, 2004). For coincubation experiments with CM overday bacterial cultures were grown in LB media up to an OD_{600} = 1 at 37 °C on a shaker and diluted to $1x10^8$ CFU/ml in IMDM with 10% FCS. Subsequently, the suspensions were centrifuged and the supernatants filtrated sterile. In contrast, for coculture experiments with inactivated bacterial pellets, overday cultures were diluted to $4x10^6$ CFU/ml, respective to the MOI of 1 used for standard bacterial coculture experiments in this study. Bacterial suspensions were again incubated for approximately 5 hours. An aliquot was taken at an OD_{600} = 0.5 and centrifuged. The bacterial pellet was fixed in 4% paraformaldehyde for one hour at room temperature, washed four times with PBS, redissolved in IMDM medium with 10% FCS and adjusted to an MOI of 2. Aliquots of the CM and fixed bacteria were plated onto Müller-Hinton-agar to test for sterility. Caco-2 cells were incubated with inactivated bacteria or CM for 6 hours, respectively.

4. Animal experiments

Conventional BALB/c mice were obtained from Harlan (Borchen, Germany). A total of 9 (6-8 weeks old) mice were used for the tissue coculture experiments. The animal experiments reported here were permitted from the district authority of Braunschweig and were conducted according to the German animal protection law.

Gnotobiotic BALB/c mice were kindly provided by André Bleich (Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School). Pelleted irradiated diet (SSNIFF© Spezialitäten, Soest, Germany) containing 22% protein, 4.5% fat and 3.9% fibre, and autoclaved distilled water were provided *ad libitum*. For the colonization experiments a total of 18 (6-8 weeks old) mice were taken from a colony maintained germ-free in plastic film isolators (Metall + Plastik,

Radolfzell, Germany) (fig. 38) and placed in gnotocages (Nunc) with sterile litter (Rettenmaier & Sons, Rosenberg, Germany) and custom-made filter caps. Handling of these animals outside the isolators/gnotocages was done under clean benches. All materials used have been sterilized by autoclaving, peracetic acid or ethanol.

Figure 38. Isolators for germ-free breeding of mice.

4.1 Bacterial colonization of gnotobiotic BALB/c mice

Gnotobiotic mice were colonized by oral application with $1x10^9$ CFU EcN or *E. coli* MG1655. Bacteria were grown overday in LB media up to OD_{600} = 1 (~ $1x10^9$ CFU/ml) and 1 ml of the culture was centrifuged for 3 min. The pellet was redissolved in 200 µl sterile PBS and given by oral gavage. After three days application of bacteria was repeated to ensure stable colonization. Colonization was controlled during the experiment by plating mouse feces on LB agar plates. Therefore, feces were weighed and adjusted with LB media to a final concentration of 300 mg/ml. They were dissolved and 10-fold dilution series were plated onto LB agar for bacterial count.

4.2 Tissue coculture

Coculture was done as described by Cima *et al.* (Cima *et al.*, 2004) with slight modifications. Small intestines were dissected, rinsed with sterile PBS and opened logitudinally. Then 5 mm pieces were cut and washed with PBS/1 mM dithiothreitol (DTT) for 15 min at 37°C on a shaker to remove excess mucus. Tissue pieces were further washed with PBS followed by two washing steps with Hanks' balanced salt solution (HBSS)/2% FCS. 3 – 5 tissue pieces per well were placed into 24 well plates in IMDM containing 10% FCS without antibiotics. Bacteria were added at $1x10^6$ CFU and cocultured for 6 hours, respectively.

5. Isolation of IECs

IECs were isolated as described elsewhere (Rogler *et al.*, 1998). Briefly, small intestine was isolated, rinsed with PBS and opened longitudinally. Mucus was removed by treatment with 1 mM DTT for 15 min at 37°C on a shaker. After washing with PBS the mucosa was placed in calcium and magnesium free HBSS containing 1.5 mM EDTA and tumbled for 10 min at 37°C. The supernatant, mainly containing debris and villus cells, was collected. The remaining mucosa was transferred to a new falcon tube and vortexed in PBS. This supernatant, now containing complete crypts, single cells, and a small amount of debris, was also collected. Pooled IECs were centrifuged with HBSS/PBS for 10 min at 1200 rpm. The pellet was redissolved in FACS buffer (PBS + 2% FCS + 2 mM EDTA) and stained for 10 min at 4°C with anti-CD45 APC antibody (BD Biosciences) on a shaker. The cells were washed again with FACS buffer and sorted with a MoFlow cell sorter (Cytomation).

6. RNA isolation and cDNA synthesis

RNA from cells and tissue was extracted using TriFast FL (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. Briefly, 750 µl TriFastFL (Peqlab) was added directly to the cells or added to the tissues and adjusted to 1ml with sterile H_2O. 100 µl of 1-Bromo-3-Chloro-Propane (BCP) were added and the suspension was incubated at room temperature for 15 min with vigorous agitation. Phase-separation was achieved by centrifugation (14.000xg, 15 min, 4°C). The RNA-containing aqueous phase was precipitated by addition of 1 volume isopropanol. After incubation at room temperature for 15 min, the RNA was centrifuged as described above, washed twice with 75% ethanol, resuspended in RNase-free water

and followed by DNase I digestion with DNA-free® (Ambion, Huntingdon Cambridgeshire, UK). For quantification of RNA, UV-absorption at 260 nm was determined using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

RNA of sorted IECs was extracted using RNeasy Minikit (Qiagen, Hilden, Germany) with on-column DNase digestion using RNase-Free DNase set (Qiagen). The IECs were therefore disrupted by addition of RLT buffer and homogenized by vortexing. Precipitation was performed by adding 75% ethanol to the lysate. This lysate was added to a RNeasy mini column in a collection tube and centrifuged subsequently. The column was washed with RW1 buffer followed by DNase digestion. After additional washing steps with RW1 and RPE buffer, RNA was eluated with RNase-free water.

RNA samples were reversely transcribed with 200 U Superscript II® (Invitrogen) for 45 min at 42°C in 20 µl assays containing 1 µl Oligo dT-primers/random hexamer primers (1:1) (Invitrogen) and 10 mM deoxynucleotide triphosphates (dNTPs).

7. RT-PCR

Conventional PCR was performed with AmpliTaq Gold® DNA polymerase (Applied Biosystems, Darmstadt, Germany) in 20 µl assays containing 0.25 pmol primers and 0.5 mM dNTPs using the following cycling conditions in a PCR thermo cycler (Biorad, München, Germany): Activation of Taq polymerase for 10 min at 95°C, followed by a first round of 10 cycles each consisting of a 30 second denaturing interval at 95°C, a 90 second annealing step at the respective primer T_m and a primer extension at 72°C for again 90 seconds. This first amplification step was followed by a second round of 27 cycles, consisting of 15 seconds denaturation, 45 seconds annealing and 90 seconds extension. PCR products were separated in 2% agarose gels and visualized by ethidium-bromide staining. For RPS9 expression a shorter PCR program was used: 94°C for 10 min followed by amplification. Each cycle of amplification included 45 seconds denaturation at 94°C, 45 seconds annealing at 58°C or 55°C and 1 min synthesis at 72°C. 30 amplification cycles were performed. Unspecific amplification products were not detected in any of the PCR runs. All primers used in this study are listed in table 8.

Table 8. Primers used for realtime RT-PCR.

Product	5' Sequence 3'	Sense/antisense	Product length, bp	T_m,°C
BNIP3 hs	GCC CAC CTC GCT CGC AGA CAC	Sense	185	55.5
	AAT ATT TTC CGG CCG ACT TGA CCA	Antisense		
CD9 hs	GGG CTG CTG CGG GGC TGT G	Sense	192	59.6
	CGC TGG GGC TCA TCC TTG GTT TTC	Antisense		
DUSP5 hs	AGC CCC AAG AGC AAC TGT GAT T	Sense	163	54.3
	AGT CCC GAG AAC CTA CCC TGA G	Antisense		
ELF3 hs	GTC AGC AAG CCA GCC CCT ACC AC	Sense	202	62.1
	GGA TCC CCC TTC TTG CAG TCA CGA	Antisense		
EMP3 hs	GCG AGA ATG GCT GGC TGA AG	Sense	204	58.3
	GGA TCT CCT CGG CGT GAA TG	Antisense		
ID2 hs	CAT CCC CCA GAA CAA GAA GGT GAG	Sense	177	58.4
	CTG ATA TCC GTG TTG AGG GTG GTC	Antisense		
ILF3 hs	GGG GCC TCC TTT CAT CCT	Sense	144	55.5
	AAA GAA GGG CAA CAG ACT ACA CGA	Antisense		
MCP-1 hs	GTC TCT GCC GCC CTT CTG TG	Sense	94	55.9
	AGG TGA CTG GGG CAT TGA TTG	Antisense		
MIP-2α hs	TTT TAG GTC AAA CCC AAG TTA GTT	Sense	150	49.0
	TTC TTG GAT TCC TCA GCC TCT ATC	Antisense		
MIP-2β hs	AAG AAG CTT ATC AGC GTA TCA T	Sense	150	50.6
	AAT AAG TAG AAC CCT CGT AAG AAA	Antisense		
NDRG1 hs	CAT TTT GGC TCA CCG TGG ATT TTC	Sense	168	57.5
	TGG GCG GCA GGT AAC GAG TCA TT	Antisense		
NFκBIA hs	CGC CCA AGC ACC CGG ATA CAG C	Sense	193	58.7
	TTC AGC CCC TTT GCA CTC ATA ACG	Antisense		
NF-κB1 hs	CCA GCT GGC AGG TAT TTG ACA T	Sense	172	53.3
	TGC GCC AGA GTA GCC CAG TTT	Antisense		
NF-κB2 hs	AAA TCT CCG GGG GCA TCA AAC C	Sense	159	61.7
	AAA GGC CTG CCA TCC ATT CTC ATC	Antisense		
PRDX4 hs	GCT TCT GCT GCC GCT ACT GCT GTT	Sense	202	61.8
	GTA GGG CGC TGG CTT GGA AAT CTT	Antisense		
RPS9 hs	CGC AGG CGC AGA CGG TGG AAG C	Sense	92	61.1
	CGA AGG GTC TCC GCG GGG TCA CAT	Antisense		
RPS9 mm	CTG GAC GAG GGC AAG ATG AAG C	Sense	143	57.5
	TGA CGT TGG CGG ATG AGC ACA	Antisense		
SFN hs	TGC CGC CCA CCA ACC CCA TCC	Sense	234	63.3
	CCC CCT CTT CCC CGG CGT TGT C	Antisense		
ZO-1 hs	ATG GCC CTG GCC TAG CAT ACG	Sense	234	54.0
	GAG GCC ATG GAA CCA GTC TCA CAT	Antisense		

Hs, human; mm, mouse; BNIP3, BCL2/adenovirus E1B interacting protein 2; CD9, CD9 antigen (p24); DUSP5 indicates dual specificity phosphatase 5; ELF3, E74-like factor 3 (ets domain transcription factor, epithelial-specific); EMP3, epithelial membrane protein 3; ID2, inhibitor of DNA binding 2 (dominant negative helix-loop-helix protein); ILF3, interleukin enhancer binding factor 3; NDRG1, N-myc downstream regulated gene1; NF-κB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105/p50); NF-κB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100); NFκBIA, nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha; MCP-1, chemoattractant protein-1 ligand 2; MIP-2α, macrophage inflammatory protein-2 alpha; MIP-2β, macrophage inflammatory protein-2 beta; PRDX4, peroxiredoxin 4; RPS9, ribosomal protein S9; SFN, stratifin; ZO-1, tight junction protein1; TNFαIP3, tumor necrosis factor, alpha-induced protein 3; VEGF, vascular growth factor and Tm, melting temperature.

Table 8. continued.

Product	5' Sequence 3'	Sense/antisense	Product length, bp	T_m,°C
ZO-1 mm	TTT TTG ACA GGG GGA GTG G	Sense	227	52.0
	TGC TGC AGA GGT CAA AGT TCA AG	Antisense		
TNFαIP3 hs	ATT GGC CTC TTT GAT ACA CTT TTG	Sense	201	52.9
	CTC ATC CCT GCT CCT TCC CTA TCT	Antisense		
VEGF hs	AAG GAG GAG GGC AGA ATC ATC ACG	Sense	192	58.8
	CAC ACT CCA GGC CCT CGT CAT TG	Antisense		

Hs, human; mm, mouse; BNIP3, BCL2/adenovirus E1B interacting protein 2; CD9, CD9 antigen (p24); DUSP5 indicates dual specificity phosphatase 5; ELF3, E74-like factor 3 (ets domain transcription factor, epithelial-specific); EMP3, epithelial membrane protein 3; ID2, inhibitor of DNA binding 2 (dominant negative helix-loop-helix protein); ILF3, interleukin enhancer binding factor 3; NDRG1, N-myc downstream regulated gene1; NF-κB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105/p50); NF-κB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100); NFκBIA, nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha; MCP-1, chemoattractant protein-1 ligand 2; MIP-2α, macrophage inflammatory protein-2 alpha; MIP-2β, macrophage inflammatory protein-2 beta; PRDX4, peroxiredoxin 4; RPS9, ribosomal protein S9; SFN, stratifin; ZO-1, tight junction protein1; TNFαIP3, tumor necrosis factor, alpha-induced protein 3; VEGF, vascular growth factor and Tm, melting temperature.

8. DNA microarray hybridization

Quality and integrity of the total RNA isolated from $1x10^6$ cells was controlled by running all samples on an Agilent 2100 Bioanalyzer (Agilent Technologies). For biotinylated target synthesis, RNA was labeled using standard protocols supplied by the manufacturer (Affymetrix, Santa Clara, CA, USA). Briefly, 5 µg total RNA was converted to dsDNA using 100 pmol of a T7T23V primer (Eurogentec, Seraing, Belgium) containing a T7 promoter. The cDNA was then used directly in an *in vitro* transcription reaction in the presence of biotinylated nucleotides. The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 12.5 µg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix HG_U133A arrays for 16 hours. After hybridization, the GeneChips were washed, stained with strepavidin-phycoerythrin and read using an Affymetrix GeneChip fluidic station and scanner.

9. Data analysis

Analysis of microarray data was performed using Affymetrix Microarray Suite 5.0, Affymetrix MicroDB 3.0 and the Affymetrix Data Mining Tool 3.0. For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray Suite. Results were filtered as follows: Genes are considered strongly regulated when their fold change is greater than or equal 2 or less than or equal –2, the statistical parameter for a significant change is less than

0.01 (change p-value for changes called increased) or greater than 0.99 (change p-value for changes called decreased). Additionally, the signal difference of a certain gene should be greater than 100. Genes are considered as weakly regulated when their fold change is greater than or equal 1.5 or less than or equal –1.5, the statistical parameter for a significant change is less than 0.001 or greater than 0.999 and the signal difference of a certain gene should be greater than 40. Cluster analysis was performed with the Genesis software 1.6.0 beta 1 and Microsoft Excel.

10. Realtime RT-PCR

Total RNA preparation and cDNA synthesis were performed as described for RT-PCR. Quantitative realtime RT-PCR was done with the GeneAmp 5700 Sequence Detection System (Perkin Elmer, Rodgau-Jügesheim, Germany) using Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene, Heidelberg, Germany) and specific primers optimized to amplify 90-250 bp fragments from the genes under investigation (tab. 8). SYBR Green chemistry is an alternative method used to perform realtime PCR analysis. SYBR Green is a dye that binds the minor groove of double stranded DNA resulting in increased intensity of the fluorescent emissions, which correlate with the amount of double stranded amplicons. Thus, this method facilitates a quantification of PCR products. The cycling program of the realtime RT-PCR was as follows: initial annealing for 2 min at 50°C followed by activation of the SureTaq polymerase (Stratagene) for 10 min at 95°C. Then, 40 amplification cycles of a 15-second denaturing interval at 95°C and a one-min annealing step at the respective primer T_m were run. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for every gene. Relative mRNA levels were determined by using included standard curves for each individual gene and further normalization to the housekeeping gene RPS9. Melting curves established the purity of the amplified band.

11. Cytokine analysis by CBA

Quantification of cytokines was performed using the CBA Human Chemokine Kit I (BD Biosciences). The CBA consists of a series of spectrally discrete particles that can be used to detect soluble analytes by flow cytometry. Briefly, polystyrene beads (7.5 µm diameter) are dyed to 5 different fluorescence intensities, which have an emission wavelength of approximately 650 nm (FL-3). Each group carries antibodies

against one of the 5 following cytokines: IL-8, RANTES, MIG, MCP-1 and IP-10, representing an unique population in FL-3 intensity. The antibody (Ab)-labeled particles serve as capture for a given cytokine in the immunoassay panel and can be identified simultaneously in a mixture. The captured cytokines are then detected using 5 specific antibodies coupled to phycoerythrin (PE), which emits its fluorescence at approximately 585 nm (FL-2). Predefined mixtures of all 5 cytokines served as calibrators (standards ranging from 0 to 5000 pg/ml) for the assay system. Added to a mixture of each 50 µl capture Ab-bead reagent and detector Ab-PE reagent was 50 µl of sample or cytokine standard. The mixture (150 µl) was incubated for 3 hours at room temperature and washed to remove unbound detector Ab-PE reagent before data acquisition using flow cytometry. Using a FACSCalibur® flow cytometer (BD Biosciences), 2-color flow cytometric analysis was performed. Acquired data obtained from 3 independent experiments were analyzed using Becton Dickinson Cytometric Bead Array software.

12. Field emission scanning electron microscopy

Caco-2 cells were seeded onto 22 mm Biocoat Collagen I coated coverslips (BD Biosciences) in six well plates and grown until confluence as described above. After coincubation, cells were washed three times with PBS and fixed with a fixation solution containing 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9), washed with cacodylate buffer and then with TE-buffer (10 mM TRIS, 2 mM EDTA, pH 6.9). Finally, cells were scratched from the wells, pelleted and embedded in 2% aqueous agar before dehydrating with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 30 min at each step. Samples in 100% acetone were allowed to reach room temperature before another change of 100% acetone. They were then subjected to critical-point drying with liquid CO_2 (CPD 030, BAL-TEC, Schalksmühle, Germany). The dried cells were covered with a gold film of about 10 nm by sputter coating (SCD040, BAL-TEC) before examination in a field emission scanning electron microscope Zeiss DSM 982 Gemini (Carl Zeiss, Oberkochen, Germany) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV. Data were stored digitally on MO-disks.

13. Western blot analysis

To examine the ZO-1 expression lysates of sorted IECs from colonized gnotobiotic mice and cocultured Lovo cells were homogenized and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), separating denaturated proteins by mass. In contrast to the small size of β Actin of approximately 40 kDa, the ZO-1 protein is relatively large with 210 - 225 kDa. Thus, ZO-1 protein expression analyses were performed using 6% SDS gels, whereas 10% SDS gels were used for β Actin protein expression analysis. Gel electrophoresis was followed by blotting the proteins on a PVDF membrane for 1.15 hours at 120 mA (β Actin) and 2.5 hours at 140 mA (ZO-1), respectively. After blocking of unsaturated protein binding sites, the membrane was incubated with the primary antibodies mouse anti-ZO-1 or rabbit anti-ZO-1 (Zymed, South San Franscisco, CA, USA) or rabbit-anti-β Actin (Sigma-Aldrich) and then subsequently with the secondary antibodies goat-anti mouse IgG and goat-anti-rabbit IgG (Dianova, Hamburg, Germany), respectively. A lysate of untreated Caco-2 cells served as positive control. Detection was performed by using ECL™ Western Blotting Detection Reagents and Amersham high performance chemiluminescence films (Amersham Biosciences, Buckinghamshire, UK).

14. Transfection

The complete coding region of human ZO-1 cDNA was subcloned into an enhanced GFP vector (pEGFP; Clontech, Basel, Switzerland) and the corresponding plasmid pZO-1EGFP-C1 was kindly provided by Heidi Wunderli-Allenspach (Department of Applied Bioscience, Swiss Federal Institute of Technology, Zürich, Switzerland) (Riesen *et al.*, 2002) (fig. 39).

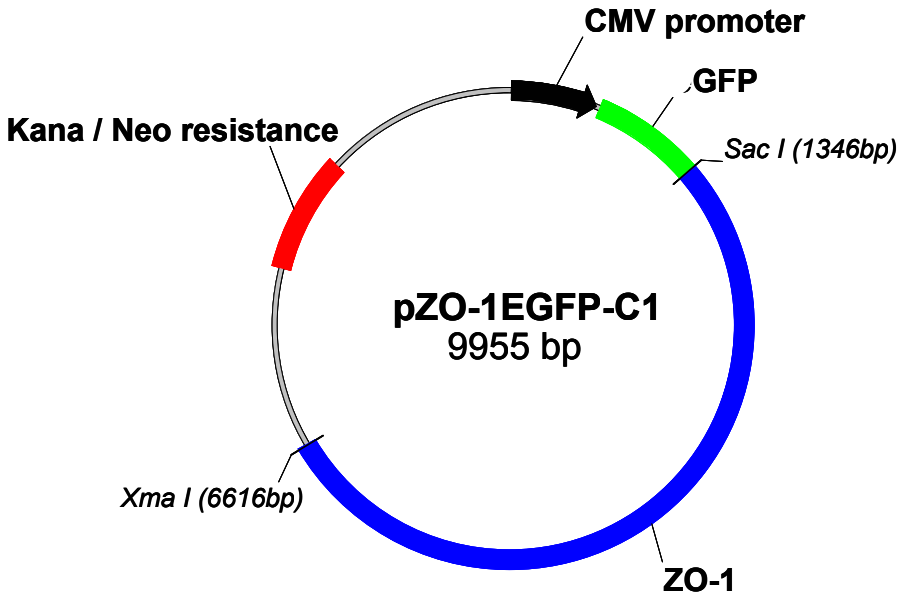

Figure 39. Plasmid map of pZO-1EGFP-C1.
pEGFP-C1 (4700 bp) encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488 nm; emission maximum = 507 nm.). A neomycin resistance cassette, consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5 (Kana/Neo), and polyadenylation signals from the Herpes simplex thymidine kinase gene, allows stably transfected eukaryotic cells to be selected using G418. CMV indicates human cytomegalovirus immediate early promoter. To retain the fluorescent properties of the native protein, a C-terminal fusion of ZO-1 with enhanced green fluorescent protein was constructed. The human cDNA of ZO-1 was therefore subcloned after restriction enzyme digestion with *XmaI* and *SacI* into the pEGFP-C1 vector. The ZO-1 construct with GFP was in the same reading frame as GFP with no interventing stop codons.

The pZO-1EGFP-C1 was transformed into *E. coli* TOP10 by electroporation and positive clones were used for subsequent isolation of plasmid DNA with the NucleoSpin®Plasmid Kit according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany).

Stable transfections were carried out by calciumphosphate coprecipitation. 50-70% confluent Lovo, cells seeded in 6 well plates, were used for subsequent transfection. Briefly, 3 hours before transfection cell culture media were replaced. 3 µg plasmid DNA ZO-1EGFP-C1, 2 µg carrier DNA and 2.5 M $CaCl_2$ were mixed and added with sterile water to a final volume of 375 µl. After incubation for 5 min at room temperature, the respective volume of 2x HEBS buffer was added drop by drop with constant mixing. This solution was then incubated for 1 hour at room temperature and the precipitate was added to the cells. 48 h post transfection positive clones were selected using 650 µg/ml G418 (Biochrom AG, Berlin, Germany) and expanded

over a period of 3 weeks. GFP positive clones were sorted by using a MoFlow cell sorter (Cytomation) and enriched ZO-1/GFP positive cells were cultured further under selective conditions. ZO-1/GFP transfected cells were analyzed in Western blot experiments and by FACS analysis for expression of the construct.

15. Invasion assay

ZO-1/GFP transfected Lovo cells were grown under selective conditions and non-transfected cells under normal conditions until confluence. Cells were washed extensively with sterile PBS and cocultured with EHEC126814, EHEC86-24 and *S. typhimurium* aro A with an MOI of 1 for 6 hours. Subsequently, cells were washed four times and extracellular bacteria were killed by addition of IMDM medium containing 10% FCS (PAA), 200 µg/ml gentamicin (Sigma-Aldrich) and 500 µg/ml penicillin/streptomycin (Invitrogen) during a 1,5 h incubation period. After four washing steps, intracellular bacteria were enumerated by plate count after Triton X-100 lysis of the cells. Relative invasion of bacteria was expressed as percentage of the invasiveness in non-transfected cells, which was 100% by definition.

16. Immunohistochemistry

Tissue sections were fixed with 4% paraformaldehyde, washed extensively and blocked with porcine serum. Subsequently, the sections were incubated with the primary antibody rabbit anti-ZO-1 (Zymed) and, after several washing steps, were then incubated with the secondary antibody Cy3 AffiniPure goat anti-rabbit IgG (Jackson Immunoresearch, Cambridgeshire, UK). The sections were then dried, covered with gelatine and visualized by fluorescence microscopy.

CHAPTER V

Supplement

V. Supplement

1. Abbreviations

APC	antigen presenting cell
bp	base pairs
CCL	chemokine ligand
CCR	chemokine receptor
cDNA	copy desoxyribonucleic acid
CFU	colony forming units
CM	conditioned media
CpG	cytosine-guanine
Ctrl	control
CXCL	chemokine ligand
CXCR	chemokine receptor
DANN	desoxyribonucleic acid
DC	dendritic cell
DTT	dithiothreitol
EcN	*E. coli* Nissle 1917
EDTA	ethylene-diamine-tetraacetic acid
FACS	fluorescence activated cell scan
FCS	fetal calf serum
GALT	gut-associated lymphoid tissue
HBSS	Hank´s Balanced Salt Solution
HEBS	Hepes buffered saline
IEC	intestinal epithelial cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
l	liter
IMDM	Iscove's Modified Dulbecco's Medium
kb	kilo base
kDA	kilo Dalton
LPS	lipopolysaccharid
µ	micro

m	milli
M	molar
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MOI	multiplicity of infection
NK	natural killer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcribed polymerase chain reaction
TCR	T cell receptor
TGF	tumor growth factor
T_H	T helper
T_C	T cytotoxic
TJ	tight junction
TNF	tumor necrosis factor
ZO-1	zonula occludens 1 (= tight junction protein 1)

2. List of figures

3. List of tables

CHAPTER VI

References

VI. References

Abreu MT, Fukata M, and Arditi M (2005) TLR signaling in the gut in health and disease. *J Immunol*, 174(8): 4453-4460.

Allavena P, Bianchi G, Zhou D, van DJ, Jilek P, Sozzani S, and Mantovani A (1994) Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. *Eur J Immunol*, 24(12): 3233-3236.

Altenhoefer A, Oswald S, Sonnenborn U, Enders C, Schulze J, Hacker J, and Oelschlaeger TA (2004) The probiotic *Escherichia coli* strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. *Fems Immunol Med Microbiol*, 40(3): 223-229.

Andreeva AY, Krause E, Muller EC, Blasig IE, and Utepbergenov DI (2001) Protein kinase C regulates the phosphorylation and cellular localization of occludin. *J Biol Chem*, 276(42): 38480-38486.

Antonetti DA, Wolpert EB, Demaio L, Harhaj NS, and Scaduto RC (2002) Hydrocortisone decreases retinal endothelial cell water and solute flux coincident with increased content and decreased phosphorylation of occludin. *J Neurochem*, 80(4): 667-677.

Araya M, Gopal P, Lindgren SE, Lodi R, Oliver G, Saxelin M, Servin AL, and Stanton C (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization. 1-34. *WHO/FAO Report*.

Ayabe T, Ashida T, Kohgo Y, and Kono T (2004) The role of Paneth cells and their antimicrobial peptides in innate host defense. *Trends Microbiol*, 12(8): 394-398.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, and Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A*, 101(44): 15718-15723.

Balda MS and Anderson JM (1993) Two classes of tight junctions are revealed by ZO-1 isoforms. *Am J Physiol*, 264(4 pt 1): 918-924.

Balda MS and Matter K (2003) Epithelial cell adhesion and the regulation of gene expression. *Trends Cell Biol*, 13(6): 310-318.

Bambou JC, Giraud A, Menard S, Begue B, Rakotobe S, Heyman M, Taddei F, Cerf-Bensussan N, and Gaboriau-Routhiau V (2004) In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli* strain. *J Biol Chem*, 279(41): 42984-42992.

Barmeyer C, Harren M, Schmitz H, Heinzel-Pleines U, Mankertz J, Seidler U, Horak I, Wiedenmann B, Fromm M, and Schulzke JD (2004) Mechanisms of diarrhea in the interleukin-2-deficient mouse model of colonic inflammation. *Am J Physiol Gastrointest Liver Physiol*, 286(2): 244-252.

Banks C, Bateman A, Payne R, Johnson P, and Sheron N (2003) Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J Pathol*, 199(1): 28-35.

Bengmark S (1998) Ecological control of the gastrointestinal tract. The role of probiotic flora. *Gut*, 42(1): 2-7.

Berglund JJ, Riegler M, Zolotarevsky Y, Wenzl E, and Turner JR (2001) Regulation of human jejunal transmucosal resistance and MLC phosphorylation by Na+-glucose cotransport. *Am J Physiol Gastrointest Liver Physiol*, 281(6): 1487-1493.

Berkes J, Viswanathan VK, Savkovic SD, and Hecht G (2003) Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut*, 52(3): 439-451.

Bernet MF, Brassart D, Neeser JR, and Servin AL (1994) *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut*, 35(4): 483-489.

Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De SC, and Sartor RB (2005) VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol*, 100(7): 1539-1546.

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, and Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science, 277(5331): 1453-1474.

Blum G, Marre R, and Hacker J (1995) Properties of *Escherichia coli* strains of serotype O6. *Infection*, 23(4): 234-236.

Blumberg RS, Lencer WI, Zhu X, Kim HS, Claypool S, Balk SP, Saubermann LJ, and Colgan SP (1999) Antigen presentation by intestinal epithelial cells. *Immunol Lett*, 69(1): 7-11.

Boquet P (2001) The cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli*. *Toxicon*, 39(11): 1673-1680.

Borriello SP (1998) Pathogenesis of *Clostridium difficile* infection. *J Antimicrob Chemother*, 41 (Suppl.): 13-19.

Borruel N, Casellas F, Antolin M, Llopis M, Carol M, Espiin E, Naval J, Guarner F, and Malagelada JR (2003) Effects of nonpathogenic bacteria on cytokine secretion by human intestinal mucosa. *Am J Gastroenterol*, 98(4): 865-870.

Boudeau J, Glasser AL, Julien S, Colombel JF, and Darfeuille-Michaud A (2003) Inhibitory effect of probiotic *Escherichia coli* strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive *E. coli* strains isolated from patients with Crohn's disease. *Aliment Pharmacol Ther*, 18(1): 45-56.

Bousvaros A, Guandalini S, Baldassano RN, Botelho C, Evans J, Ferry GD, Goldin B, Hartigan L, Kugathasan S, Levy J, Murray KE, Oliva-Hemker M, Rosh JR, Tolia V, Zholudev A, Vanderhoof JA, and Hibberd PL (2005) A randomized, double-blind trial of *Lactobacillus* GG versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflamm Bowel Dis*, 11(9): 833-839.

Boyer B and Thiery JP (1989) Epithelial cell adhesion mechanisms. *J Membr Biol*, 112(2): 97-108.

Brand S, Olszak T, Dambacher J, Beigel F, Otte JM, Goke B, and Eichhorst ST (2005) Intestinal epithelial cell expressed Ccr6 mediates epithelial cell restitution and cell proliferation. *Gastroenterology*, 128(4): 142.

Butler JE, Weber P, Sinkora M, Sun J, Ford SJ, and Christenson RK (2000) Antibody repertoire development in fetal and neonatal piglets. II. Characterization of heavy chain complementarity-determining region 3 diversity in the developing fetus. *J Immunol*, 165(12): 6999-7010.

Campieri M and Gionchetti P (1999) Probiotics in inflammatory bowel disease: New insight to pathogenesis or a possible therapeutic alternative? *Gastroenterology*, 116(5): 1246-1249.

Candela M, Seibold G, Vitali B, Lachenmaier S, Eikmanns BJ, and Brigidi P (2005) Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. *Res Microbiol*, 156(8): 887-895.

Carr MW, Roth SJ, Luther E, Rose SS, and Springer TA (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A*, 91(9): 3652-3656.

Castagliuolo I, Keates AC, Wang CC, Pasha A, Valenick L, Kelly CP, Nikulasson ST, LaMont JT, and Pothoulakis C (1998) *Clostridium difficile* toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. *J Immunol*, 160(12): 6039-6045.

Cebra JJ, Periwal SB, Lee G, Lee F, and Shroff KE (1998) Development and maintenance of the gut-associated lymphoid tissue (GALT): the roles of enteric bacteria and viruses. *Dev Immunol*, 6(1-2): 13-18.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nunez G, and Inohara N (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol*, 4(7): 702-707.

Ciacci C, Lind SE, and Podolsky DK (1993) Transforming growth factor beta regulation of migration in wounded rat intestinal epithelial monolayers. *Gastroenterology*, 105(1): 93-101.

Cima I, Corazza N, Dick B, Fuhrer A, Herren S, Jakob S, Ayuni E, Mueller C, and Brunner T (2004) Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. *J Exp Med*, 20;200(12): 1635-1646.

Clemente MG, De Virgiliis S, Kang JS, Macatagney R, Musu MP, Di Pierro MR, Drago S, Congia M, and Fasano A (2003) Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut*, 52(2): 218-223.

Coconnier MH, Bernet MF, Kerneis S, Chauviere G, Fourniat J, and Servin AL (1993) Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. *Fems Microbiol Lett*, 110(3): 299-305.

Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, and Maniatis T (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *Faseb J*, 9(10): 899-909.

Crabbe PA, Bazin H, Eyssen H, and Heremans JF (1968) The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. The germ-free intestinal tract. Int *Arch Allergy Appl Immunol*, 34(4): 362-375.

Crabtree JE and Farmery SM (1995) *Helicobacter pylori* and gastric mucosal cytokines: evidence that CagA-positive strains are more virulent. *Lab Invest*, 73(6): 742-745.

Crohn BB (1967) Granulomatous diseases of the small and large bowel. A historical survey. *Gastroenterology*, 52(5): 767-772.

Cruchet S, Obregon MC, Salazar G, Diaz E, and Gotteland M (2003) Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii* La1 on *Helicobacter pylori* colonization in children. *Nutrition*, 19(9): 716-721.

Cui HH, Chen CL, Wang JD, Yang YJ, Cun Y, Wu JB, Liu YH, Dan HL, Jian YT, and Chen XQ (2004) Effects of probiotic on intestinal mucosa of patients with ulcerative colitis. *World J Gastroenterol*, 10(10): 1521-1525.

Dahinden CA, Geiser T, Brunner T, von T, V, Caput D, Ferrara P, Minty A, and Baggiolini M (1994) Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J Exp Med*, 179(2): 751-756.

Dalmasso G, Loubat A, Dahan S, Calle G, Rampal P, and Czerucka D (2006) *Saccharomyces boulardii* prevents TNF-alpha-induced apoptosis in EHEC-infected T84 cells. *Res Microbiol*, Jan 13 (epub ahead of print).

Danese S, Sans M, and Fiocchi C (2004) Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev*, 3(5): 394-400.

Dean P and Kenny B (2004) Intestinal barrier dysfunction by enteropathogenic *Escherichia coli* is mediated by two effector molecules and a bacterial surface protein. *Mol Microbiol*, 54(3): 665-675.

Demaio L, Chang YS, Gardner TW, Tarbell JM, and Antonetti DA (2001) Shear stress regulates occludin content and phosphorylation. *Am J Physiol Heart Circ Physiol*, 281(1): 105-113.

Dillon ST, Rubin EJ, Yakubovich M, Pothoulakis C, LaMont JT, Feig LA, and Gilbert RJ (1995) Involvement of Ras-related Rho proteins in the mechanisms of action of *Clostridium difficile* toxin A and toxin B. *Infect Immun*, 63(4): 1421-1426.

Dotan I and Rachmilewitz D (2005) Probiotics in inflammatory bowel disease: possible mechanisms of action. *Curr Opin Gastroenterol*, 21(4): 426-430.

Doyle SE, McIvor WE, and Menaker M (2002) Circadian rhythmicity in dopamine content of mammalian retina: role of the photoreceptors. *J Neurochem*, 83(1): 211-219.

Drakes M, Blanchard T, and Czinn S (2004) Bacterial probiotic modulation of dendritic cells. *Infect Immun*, 72(6): 3299-3309.

Dwinell MB, Eckmann L, Leopard JD, Varki NM, and Kagnoff MF (1999) Chemokine receptor expression by human intestinal epithelial cells. *Gastroenterology*, 117(2): 359-367.

Dwinell MB, Lugering N, Eckmann L, and Kagnoff MF (2001) Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. *Gastroenterology*, 120(1): 49-59.

Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, and Kagnoff MF (1999) NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacterial. *J Immunol*, 163(3): 1457-1466.

Elmes ME, Stanton MR, Howells CH, and Lowe GH (1984) Relation between the mucosal flora and Paneth cell population of human jejunum and ileum. *J Clin Pathol*, 37(11): 1268-1271.

Falk PG, Hooper LV, Midtvedt T, and Gordon JI (1998) Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev*, 62(4): 1157-1170.

Faller A, Schuenke M (1995) Der Körper des Menschen, *Thieme*, Stuttgart: 263.

Fasano A and Nataro JP (2004) Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins. *Adv Drug Deliv Rev*, 56(6): 795-807.

Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A, and Goldblum SE (2000) Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet*, 355(9214): 1518-1519.

Fedorak RN, Gionchetti P, Campieri M, Madsen K, Isaacs K, Desimone C, and Sartor B (2003) VSL#3 Probiotic mixture induces remission in patients with active ulcerative colitis. *Gastroenterology*, 124(4): 377.

Fellermann K, Wehkamp J, Herrlinger KR, and Stange EF (2003) Crohn's disease: a defensin deficiency syndrome? *Eur J Gastroenterol Hepatol*, 15(6): 627-634.

Framson PE, Cho DH, Lee LY, and Hershberg RM (1999) Polarized expression and function of the costimulatory molecule CD58 on human intestinal epithelial cells. *Gastroenterology*, 116(5): 1054-1062.

Freter R (1981) Mechanisms of association of bacteria with mucosal surfaces. *Ciba Found Symp*, 80: 36-55.

Fuentes ME, Durham SK, Swerdel MR, Lewin AC, Barton DS, Megill JR, Bravo R, and Lira SA (1995) Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J Immunol*, 155(12): 5769-5776.

Fukata M, Michelsen KS, Eri R, Thomas LS, Hu B, Lukasek K, Nast CC, Lechago J, Xu R, Naiki Y, Soliman A, Arditi M, and Abreu MT (2005) Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol*, 288(5): 1055-1065.

Fuller R (1989) Probiotics in man and animals. *J Appl Bacteriol*, 66(5):365-378.

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, and Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol*, 123(6 Pt 2): 1777-1788.

Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, and Tsukita S (1994) Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol*, 127(6 Pt 1): 1617-1626.

Galdeano CM and Perdigon G (2004) Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J Appl Microbiol*, 97(4): 673-681.

Gewirtz AT, Navas TA, Lyons S, Godowski PJ, and Madara JL (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol*, 167(4): 1882-1885.

Gewirtz AT, Rao AS, Simon PO, Jr., Merlin D, Carnes D, Madara JL, and Neish AS (2000) *Salmonella typhimurium* induces epithelial IL-8 expression via Ca(2+)-mediated activation of the NF-kappaB pathway. *J Clin Invest*, 105(1): 79-92.

Ghosh S, May MJ, and Kopp EB (1998) NF-kappa B and rel proteins: Evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*, 16: 225-60.

Giatromanolaki A, Sivridis E, Maltezos E, Papazoglou D, Simopoulos C, Gatter KC, Harris AL, and Koukourakis MI (2003) Hypoxia inducible factor 1 alpha and 2 alpha overexpression in inflammatory bowel disease. *J Clin Pathol*, 56(3): 209-213.

Gionchetti P, Lammers KM, Rizzello F, and Campieri M (2005) Probiotics and barrier function in colitis. *Gu*t, 54(7): 898-900.

Gionchetti P, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, and Campieri M (2003) Prophylaxis of pouchitis onset with probiotic therapy: A double-blind, placebo-controlled trial. *Gastroenterology*, 124(5): 1202-1209.

Girardin SE, Boneca IG, Carneiro LAM, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, Coyle AJ, Bertin J, Sansonetti PJ, and Philpott DJ (2003a) Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science*, 300(5625): 1584-1587.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, and Sansonetti PJ (2003b) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem*, 278(11): 8869-8872.

Gon Y, Asai Y, Hashimoto S, Mizumura K, Jibiki I, Machino T, Ra C, and Horie T (2004) A20 inhibits toll-like receptor 2-and 4-mediated interleukin-8 synthesis in airway epithelial cells. *Am J Respir Cell Mol Biol*, 31(3): 330-336.

Gordon HA and Pesti L (1971) The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev*, 35(4): 390-429.

Gottardi CJ, Arpin M, Fanning AS, and Louvard D (1996) The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. *Proc Natl Acad Sci U S A*, 93(20): 10779-10784.

Greene JD and Klaenhammer TR (1994) Factors involved in adherence to lactobacilli to human Caco-2 cells. *Appl Environ Microbiol*, 60(12): 4487-4494.

Gronlund MM, Lehtonen OP, Eerola E, and Kero P (1999) Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr*, 28(1):19-25.

Grossmann J, Walther K, Artinger M, Kiessling S, Steinkamp M, Schmautz WK, Stadler F, Bataille F, Schultz M, Scholmerich J, and Rogler G (2003) Progress on isolation and short-term ex-vivo culture of highly purified non-apoptotic human intestinal epithelial cells (IEC). *Eur J Cell Biol*, 82(5): 262-270.

Grozdanov L, Raasch C, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, and Dobrindt U (2004) Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. *J Bacteriol*, 186(16): 5432-5441.

Grozdanov L, Zahringer U, Blum-Oehler G, Brade L, Henne A, Knirel YA, Schombel U, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, Rietschel ET, and Dobrindt U (2002) A single nucleotide exchange in the wzy gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of *Escherichia coli* strain Nissle 1917. *J Bacteriol*, 184(21): 5912-5925.

Gu L, Tseng S, Horner RM, Tam C, Loda M, and Rollins BJ (2000) Control of T(H)2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature*, 404(6776): 407-411.

Guandalini S (2002) Use of *Lactobacillus* GG in paediatric Crohn's disease. *Dig Liver Dis*, 34(Suppl. 2): 63-65.

Guandalini S, Pensabene L, bu Zikri M, Dias JA, Casali LG, Hoekstra H, Kolacek S, Massar K, Micetic-Turk D, Papadopoulou A, de Sousa JS, Sandhu B, Szajewska H, and Weizman Z (2000) *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhea: A multicenter European trial. *J Pediatr Gastroenterol Nutr*, 30(1): 54-60.

Guarner F, Casellas F, Borruel N, Antolin M, Videla S, Vilaseca J, and Malagelada JR (2002) Role of microecology in chronic inflammatory bowel diseases. *Eur J Clin Nutr*, Suppl. 4: 634-638.

Guarner F and Schaafsma GJ (1998) Probiotics. *Int J Food Microbiol*, 39(3): 237-238.

Gumbiner B, Lowenkopf T, and Apatira D (1991) Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci U S A*, 88(8): 3460-3464.

Gunn MD, Nelken NA, Liao X, and Williams LT (1997) Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *J Immunol*, 158(1): 376-383.

Gunzer F, Hennig-Pauka I, Waldmann KH, Sandhoff R, Gröne HJ, Kreipe HH, Matussek A, and Mengel M (2002) Gnotobiotic piglets develop thrombotic microangiopathy after oral infection with enterohemorrhagic *Escherichia coli*. *Am J Clin Pathol*, 118(3): 364-375.

Gupta P, Andrew H, Kirschner BS, and Guandalini S (2000) Is *Lactobacillus* GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J Pediatr Gastroenterol Nutr*, 31(4): 453-457.

Guslandi M, Giollo P, and Testoni PA (2003) A pilot trial of *Saccharomyces boulardii* in ulcerative colitis. *Eur J Gastroenterol Hepatol*, 15(6): 697-698.

Guslandi M, Mezzi G, Sorghi M, and Testoni PA (2000) *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Dig Dis Sci*, 45(7): 1462-1464.

Haller D, Bode C, Hammes WP, Pfeifer AMA, Schiffrin EJ, and Blum S (2000) Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut*, 47(1): 79-87.

Haller D, Russo MP, Sartor RB, and Jobin C (2002) IKK beta and phosphatidylinositol 3-kinase/Akt participate in non-pathogenic Gram-negative enteric bacteria-induced RelA phosphorylation and NF-kappa B activation in both primary and intestinal epithelial cell lines. *J Biol Chem*, 277(41): 38168-38178.

Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van DS, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, and Mathew CG (2001) Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet*, 357(9272): 1925-1928.

Hao WL and Lee YK (2004) Microflora of the gastrointestinal tract: a review. *Methods Mol Biol*, 268: 491-502.

Harhaj NS and Antonetti DA (2004) Regulation of tight junctions and loss of barrier function in pathophysiology. *Int J Biochem Cell Biol*, 36(7): 1206-1237.

Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, and Welling GW (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr*, 30(1): 61-67.

Havenaar R and Huis in 't Veld JHJ (1992) Probiotics: a general view. Wood B.J.B., The Lactic Acid Bacteria, *Chapman & Hall*, New York: 209-224.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, and Aderem A (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, 410(6832): 1099-1103.

Hecht G, Pestic L, Nikcevic G, Koutsouris A, Tripuraneni J, Lorimer DD, Nowak G, Guerriero V, Elson EL, and deLanerolle P (1996) Expression of the catalytic domain of myosin light chain kinase increases paracellular permeability. *Am J Physiol*, 40(5): 1678-1684.

Helgeland L, Vaage JT, Rolstad B, Midtvedt T, and Brandtzaeg P (1996) Microbial colonization influences composition and T-cell receptor V beta repertoire of intraepithelial lymphocytes in rat intestine. *Immunology*, 89(4): 494-501.

Hentges DJ (1993) The anaerobic microflora of the human body. *Clin Infect Dis*, 16 (Suppl. 4): 175-180.

Hershberg RM and Mayer LF (2000) Antigen processing and presentation by intestinal epithelial cells - polarity and complexity. *Immunol Today*, 21(3): 123-128.

Hirano J, Yoshida T, Sugiyama T, Koide N, Mori I, and Yokochi T (2003) The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells in vitro. *Microbiol Immunol*, 47(6): 405-409.

Hobbie S, Chen LM, Davis RJ, and Galan JE (1997) Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol*, 159(11): 5550-5559.

Hollander D (1999) Intestinal permeability, leaky gut, and intestinal disorders. *Curr Gastroenterol Rep*, 1(5): 410-416.

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, and Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr*, 73(2): 365-373.

Hooper LV, Bry L, Falk PG, and Gordon JI (1998) Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. *Bioessays*, 20(4): 336-343.

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, and Gordon JI (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science*, 291(5505):881-884.

Hoshino K, Kaisho T, Iwabe T, Takeuchi O, and Akira S (2002) Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *Int Immunol*, 14(10): 1225-1231.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, and Akira S (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the Lps gene product. *J Immunol*, 162(7): 3749-3752.

Hu L and Hickey TE (2005) *Campylobacter jejuni* induces secretion of proinflammatory chemokines from human intestinal epithelial cells. *Infect Immun*, 73(7): 4437-4440.

Huang GT, Eckmann L, Savidge TC, and Kagnoff MF (1996) Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM)-1) expression and neutrophil adhesion. *J Clin Invest*, 98(2):572-583.

Ishikawa H, Akedo I, Umesaki Y, Tanaka R, Imaoka A, and Otani T (2003) Randomized controlled trial of the effect of Bifidobacteria-fermented milk on ulcerative colitis. *J Am Coll Nutr*, 22(1):56-63.

Ito CY, Adey N, Bautch VL, and Baldwin AS, Jr. (1995) Structure and evolution of the human IKBA gene. *Genomics*, 20;29(2): 490-495.

Itoh M, Furuse M, Morita K, Kubota K, Saitou M, and Tsukita S (1999) Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol*, 147(6): 1351-1363.

Ivanov AI, Nusrat A, and Parkos CA (2004) The epithelium in inflammatory bowel disease: potential role of endocytosis of junctional proteins in barrier disruption. *Novartis Found Symp*, 263: 115-124.

Jepson MA, Collares-Buzato CB, Clark MA, Hirst BH, and Simmons NL (1995) Rapid disruption of epithelial barrier function by *Salmonella typhimurium* is associated with structural modification of intercellular junctions. *Infect Immun*, 63(1): 356-359.

Jepson MA, Schlecht HB, and Collares-Buzato CB (2000) Localization of dysfunctional tight junctions in *Salmonella enterica* serovar typhimurium-infected epithelial layers. *Infect Immun*, 68(12): 7202-7208.

Jetten AM and Suter U (2000) The peripheral myelin protein 22 and epithelial membrane protein family. *Prog Nucleic Acid Res Mol Biol,* 64: 97-129.

Jiang Y, Beller DI, Frendl G, and Graves DT (1992) Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol*, 148(8): 2423-2428.

Jung HC, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E, and Kagnoff MF (1995) A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest*, 95(1): 55-65.

Kailasapathy K and Chin J (2000) Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol Cell Biol*, 78(1): 80-88.

Kalliomaki M, Salminen S, Poussa T, Arvilommi H, and Isolauri E (2003) Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet*, 361(9372):1869-1871.

Kalliomaki MA and Isolauri E (2004) Probiotics and down-regulation of the allergic response. *Immunol Allergy Clin North Am*, 24(4): 739-52.

Kamada N, Inoue N, Hisamatsu T, Okamoto S, Matsuoka K, Sato T, Chinen H, Hong KS, Yamada T, Suzuki Y, Suzuki T, Watanabe N, Tsuchimoto K, and Hibi T (2005) Nonpathogenic *Escherichia coli* strain Nissle1917 prevents murine acute and chronic colitis. *Inflamm Bowel Dis*, 11(5): 455-463.

Kawai T, Adachi O, Ogawa T, Takeda K, and Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, 11(1): 115-122.

Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, and Akira S (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*, 167(10): 5887-5894.

Kelly D, Campbell JI, King TP, Grant G, Jansson EA, Coutts AG, Pettersson S, and Conway S (2004) Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol*, 5(1): 104-112.

Kelly D, Conway S, and Aminov R (2005) Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunol*, 26(6): 326-333.

Kim JK, Takeuchi M, and Yokota Y (2004) Impairment of intestinal intraepithelial lymphocytes in Id2 deficient mice. *Gut*, 53(4): 480-486.

Kim YG, Ohta T, Takahashi T, Kushiro A, Nomoto K, Yokokura T, Okada N, and Danbara H (2006) Probiotic *Lactobacillus casei* activates innate immunity via NF-kappaB and p38 MAP kinase signaling pathways. *Microbes Infect*, Jan 18 (epub ahead of print).

Kohler K, Louvard D, and Zahraoui A (2004) Rab13 regulates PKA signaling during tight junction assembly. *J Cell Biol*, 165(2): 175-180.

Konowalchuk J, Speirs JI, and Stavric S (1977) Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun*, 18(3): 775-779.

Kontiokari T, Sundqvist K, Nuutinen M, Pokka T, Koskela M, and Uhari M (2001) Randomised trial of cranberry-lingonberry juice and *Lactobacillus* GG drink for the prevention of urinary tract infections in women. *BMJ*, 322(7302): 1571-1573.

Kothari S, Cizeau J, Millan-Ward E, Israels SJ, Bailes M, Ens K, Kirshenbaum LA, and Gibson SB (2003) BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. *Oncogene*, 22(30): 4734-4744.

Kovanen PE, Rosenwald A, Fu J, Hurt EM, Lam LT, Giltnane JM, Wright G, Staudt LM, and Leonard WJ (2003) Analysis of gamma(c)-family cytokine target genes - Identification of dual-specificity phosphatase 5 (DUSP5) as a regulator of mitogen-activated protein kinase activity in interleukin-2 signaling. *J Biol Chem*, 278(7): 5205-5213.

Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, and Schulze J (2004) Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. *Gut*, 53(11): 1617-1623.

Kruis W, Fric P, and Stolte MS (2001) Maintenance of remission in ulcerative colitis is equally effective with *Escherichia coli* Nissle 1917 and with standard mesalamine. *Gastroenterology*, 120(5): 127.

Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, and Stolte M (1997) Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther*, 11(5): 853-858.

Kucharzik T, Lugering N, Pauels HG, Domschke W, and Stoll R (1998) IL-4, IL-10 and IL-13 down-regulate monocyte-chemoattracting protein-1 (MCP-1) production in activated intestinal epithelial cells. *Clin Exp Immunol*, 111(1): 152-157.

Lammers KM, Brigidi P, Vitali B, Gionchetti P, Rizzello F, Caramelli E, Matteuzzi D, and Campieri M (2003) Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *Fems Immunol Med Microbiol*, 38(2): 165-172.

Lan JG, Cruickshank SM, Singh JC, Farrar M, Lodge JP, Felsburg PJ, and Carding SR (2005) Different cytokine response of primary colonic epithelial cells to commensal bacteria. *World J Gastroenterol*, 11(22): 3375-3384.

Lauw FN, Caffrey DR, and Golenbock DT (2005) Of mice and man: TLR11 (finally) finds profilin. *Trends Immunol*, 26(10):509-511.

Lee A and O'Rourke J (1993) Gastric bacteria other than *Helicobacter pylori*. *Gastroenterol Clin North Am*, 22(1):21-42.

Lilly DM and Stillwell RH (1965) Probiotics: Growth-promoting factors produced by microorganisms. *Science*, 147, 747-748.

Llopis M, Antolin M, Guarner F, Salas A, and Malagelada JR (2005) Mucosal colonisation with *Lactobacillus casei* mitigates barrier injury induced by exposure to trinitronbenzene sulphonic acid. *Gut*, 54(7): 955-959.

Loetscher P, Seitz M, Baggiolini M, and Moser B (1996) Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J Exp Med*, 184(2): 569-577.

Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, and Moser B (1996) Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. *J Immunol*, 156(1): 322-327.

Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, and Rollins BJ (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med*, 187(4): 601-608.

Luyer MD, Buurman WA, Hadfoune M, Speelmans G, Knol J, Jacobs JA, Dejong CH, Vriesema AJ, and Greve JW (2005) Strain-specific effects of probiotics on gut barrier integrity following hemorrhagic shock. *Infect Immun*, 73(6): 3686-3692.

Ma TY, Boivin MA, Ye DM, Pedram A, and Said HM (2005) Mechanism of tumor necrosis factor-alpha modulation of Caco-2 intestinal epithelial tight junction barrier: Role of myosin light chain kinase protein expression. *Gastroenterology*, 128(4): 539.

Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, and Said HM (2004) TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol*, 286(3): 367-376.

MacDermott RP (1999) Chemokines in the inflammatory bowel diseases. *J Clin Immunol*, 19(5): 266-272.

Mack DR, Ahrne S, Hyde L, Wei S, and Hollingsworth MA (2003) Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. *Gut*, 52(6): 827-833.

Mack DR, Michail S, Wei S, McDougall L, and Hollingsworth MA (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am J Physiol*, 276(4): 941-950.

Madara JL and Trier JS (1980) Structural abnormalities of jejunal epithelial cell membranes in celiac sprue. *Lab Invest*, 43(3): 254-261.

Madrigal-Estebas L, McManus R, Byrne B, Lynch S, Doherty DG, Kelleher D, O'Donoghue DP, Feighery C, and O'Farrelly C (1997) Human small intestinal epithelial cells secrete interleukin-7 and differentially express two different interleukin-7 mRNA Transcripts: implications for extrathymic T-cell differentiation. *Hum Immunol*, 58(2): 83-90.

Maeda S, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, Eckmann L, and Karin M (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science*, 307(5710): 734-738.

Malchow HA (1997) Crohn's disease and *Escherichia coli*. A new approach in therapy to maintain remission of colonic Crohn's disease? *J Clin Gastroenterol*, 25(4): 653-658.

Mandel L, Trebichavsky I, Splichal I, and Schulze J (1995) Stimulation of intestinal immune cells by *E. coli* in gnotobiotic piglets. *Adv Exp Med Biol*, 371A: 463-464.

Mankertz J, Tavalali S, Schmitz H, Mankertz A, Riecken EO, Fromm M, and Schulzke JD (2000) Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. *J Cell Sci*, 113 (Pt 11): 2085-2090.

Marteau PR, de Vrese M, Cellier CJ, and Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. *Am J Clin Nutr*, 73(2): 430-436.

Matsushima K, Larsen CG, DuBois GC, and Oppenheim JJ (1989) Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J Exp Med*, 169(4): 1485-1490.

Matter K and Balda MS (2003) Signalling to and from tight junctions. *Nat Rev Mol Cell Biol*, 4(3): 225-236.

Mayer L (1998) Current concepts in mucosal immunity. I. Antigen presentation in the intestine: new rules and regulations. *Am J Physiol*, 274(1 Pt 1): 7-9.

McCann ML, Abrams RS, and Nelson RP, Jr. (1994) Recolonization therapy with nonadhesive *Escherichia coli* for treatment of inflammatory bowel disease. *Ann N Y Acad Sci*, 730: 243-245.

McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, and Shanahan F (2003) Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut*, 52(7): 975-980.

McCarthy KM, Francis SA, McCormack JM, Lai J, Rogers RA, Skare IB, Lynch RD, and Schneeberger EE (2000) Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. *J Cell Science*, 113(19): 3387-3398.

McCullogh JS, Ratcliffe B, Mandir N, Carr KE, and Goodlad RA (1998) Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. *Gut*, 42(6): 799-806.

Meddings JB (1997) Review article: Intestinal permeability in Crohn's disease. *Aliment Pharmacol Ther*, 11 (Suppl. 3): 47-53.

Medzhitov R and Janeway C, Jr. (2000) The Toll receptor family and microbial recognition. *Trends Microbiol*, 8(10): 452-456.

Menard S, Candalh C, Bambou JC, Terpend K, Cerf-Bensussan N, and Heyman M (2004) Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. *Gut*, 53(6): 821-828.

Metchnikoff C (1907) Lactic acid as inhibiting intestinal putrefaction. W. Heinemann, The prolongation of life: Optimistic studies, London: 161-183.

Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, Bavari S, and Klaenhammer TR (2005) Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci U S A*, 102(8): 2880-2885.

Mollenbrink M and Bruckschen E (1994) Treatment of chronic constipation with physiologic *Escherichia coli* bacteria. Results of a clinical study of the effectiveness and tolerance of microbiological therapy with the *E. coli* Nissle 1917 strain (Mutaflor). *Med Klin* (Munich), 89(11): 587-593.

Moore WE and Moore LH (1995) Intestinal floras of populations that have a high risk of colon cancer. *Appl Environ Microbiol*, 61(9): 3202-3207.

Mowat AM (2003) Anatomical basis of tolerance and immunity to intestinal antigens. N*at Rev Immunol*, 3(4): 331-341.

Muza-Moons MM, Schneeberger EE, and Hecht GA (2004) Enteropathogenic *Escherichia coli* infection leads to appearance of aberrant tight junctions strands in the lateral membrane of intestinal epithelial cells. *Cell Microbiol*, 6(8): 783-793.

Nakazawa A, Watanabe M, Kanai T, Yajima T, Yamazaki M, Ogata H, Ishii H, Azuma M, and Hibi T (1999) Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucose. *Gastroenterology*, 117(3): 536-545.

Neish AS (2002) The gut microflora and intestinal epithelial cells: a continuing dialogue. *Microbes Infect*, 4(3): 309-317.

Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, and Madara JL (2000) Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science*, 289(5484): 1560-1563.

Neurath MF, Finotto S, and Glimcher LH (2002) The role of Th1/Th2 polarization in mucosal immunity. *Nat Med*, 8(6): 567-573.

Neutra MR, Mantis NJ, and Kraehenbuhl JP (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol*, 2(11): 1004-1009.

Nissle A (1918) Die antagonistische Behandlung chronischer Darmstörungen mit Colibakterien. *Med Klin*, 2: 29-30.

Nissle A. (1919) Weiteres über die Mutaflorbehandlung unter besonderer Berücksichtigung der chronischen Ruhr. *Münch Med Wschr*, 25: 678-681.

Nusrat A, von Eichel-Streiber C, Turner JR, Verkade P, Madara JL, and Parkos CA (2001) *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infect Immun*, 69(3): 1329-1336.

Oettgen P, Alani RM, Barcinski MA, Brown L, Akbarali Y, Boltax J, Kunsch C, Munger K, and Libermann TA (1997) Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family. *Mol Cell Biol*, 17(8): 4419-4433.

Ogawa H, Iimura M, Eckmann L, and Kagnoff MF (2004) Regulated production of the chemokine CCL28 in human colon epithelium. *Am J Physiol*, 287(5): 1062-1069.

Ohno Y, Lee J, Fusunyan RD, MacDermott RP, and Sanderson IR (1997) Macrophage inflammatory protein-2: Chromosomal regulation in rat small intestinal epithelial cells. *Proc Natl Acad Sci U S A*, 94(19): 10279-10284.

Ohtsuka Y, Lee J, Stamm DS, and Sanderson IR (2001) MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine. *Gut*, 49(4): 526-533.

Ohtsuka Y and Sanderson IR (2003) Dextran sulfate sodium-induced inflammation is enhanced by intestinal epithelial cell chemokine expression in mice. *Pediatr Res*, 53(1): 143-147.

Oostenbrug LE, van Dullemen HM, te Meerman GJ, and Jansen PL (2003) IBD and genetics: new developments. *Scand J Gastroenterol*, 239 (Suppl.): 63-68.

Otte JM and Podolsky DK (2004) Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. *Am J Physiol*, 286(4): 613-626.

Ouellette AJ and Lualdi JC (1990) A novel mouse gene family coding for cationic, cysteine-rich peptides. Regulation in small intestine and cells of myeloid origin. *J Biol Chem*, 265(17): 9831-9837.

Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, and Brenner DA (2003) Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology*, 37(5): 1043-1055.

Panja A, Siden E, and Mayer L (1995) Synthesis and regulation of accessory/proinflammatory cytokines by intestinal epithelial cells. *Clin Exp Immunol*, 100(2): 298-305.

Pantoflickova D, Corthesy-Theulaz I, Dorta G, Stolte M, Isler P, Rochat F, Enslen M, and Blum AL (2003) Favourable effect of regular intake of fermented milk containing *Lactobacillus johnsonii* on *Helicobacter pylori* associated gastritis. *Aliment Pharmacol Ther*, 18(8): 805-813.

Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R, and Rampal P (2005) *Lactobacillus casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. *Res Microbiol*, 156(2): 256-262.

Paton JC and Paton AW (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev*, 11(3): 450-479.

Patzer SI, Baquero MR, Bravo D, Moreno F, and Hantke K (2003) The colicin G, H and X determinants encode microcins M and H47, which might utilize the catecholate siderophore receptors FepA, Cir, Fiu and IroN. *Microbiology*, 149(Pt 9): 2557-2570.

Pena JA, Rogers AB, Ge ZM, Ng V, Li SY, Fox JG, and Versalovic J (2005) Probiotic *Lactobacillus* spp. diminish *Helicobacter hepaticus*-induced inflammatory bowel disease in interleukin-10-deficient mice. *Infect Immun*, 73(2): 912-920.

Petrof EO, Kojima K, Ropeleski MJ, Musch MW, Tao Y, De SC, and Chang EB (2004) Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology*, 127(5): 1474-1487.

Philpott DJ, McKay DM, Mak W, Perdue MH, and Sherman PM (1998) Signal transduction pathways involved in enterohemorrhagic *Escherichia coli*-induced alterations in T84 epithelial permeability. *Infect Immun*, 66(A): 1680-1687.

Phuapradit P, Varavithya W, Vathanophas K, Sangchai R, Podhipak A, Suthutvoravut U, Nopchinda S, Chantraruksa V, and Haschke F (1999) Reduction of rotavirus infection in children receiving Bifidobacteria-supplemented formula. *J Med Assoc Thai*, 82(Suppl. 1): 43-48.

Pirzer U, Schonhaar A, Fleischer B, Hermann E, and Meyer zum Buschenfelde KH (1991) Reactivity of infiltrating T lymphocytes with microbial antigens in Crohn's disease. *Lancet*, 338(8777): 1238-1239.

Pitman RS and Blumberg RS (2000) First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. *J Gastroenterol*, 35(11): 805-814.

Prantera C, Scribano ML, Falasco G, Andreoli A, and Luzi C (2002) Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with *Lactobacillus* GG. *Gut*, 51(3): 405-409.

Qi R, Ozaki Y, Kuroda K, Asazuma N, Yatomi Y, Satoh K, Nomura S, and Kume S (1996) Differential activation of human platelets induced by Fc gamma receptor II cross-linking and by anti-CD9 monoclonal antibody. *J Immunol*, 157(12): 5638-5645.

Qin HL, Shen TY, Gao ZG, Fan XB, Hang XM, Jiang YQ, and Zhang HZ (2005) Effect of *Lactobacillus* on the gut microflora and barrier function of the rats with abdominal infection. *World J Gastroenterol*, 11(17): 2591-2596.

Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, and Raz E (2004) Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology*, 126(2): 520-528.

Rahner C, Mitic LL, and Anderson JM (2001) Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology*, 120(2): 411-422.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, and Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*, 118(2): 229-241.

Reid G (2001) Probiotic agents to protect the urogenital tract against infection. *Am J Clin Nutr*, 73(2): 437-443.

Reinecker HC, Loh EY, Ringler DJ, Mehta A, Rombeau JL, and MacDermott RP (1995) Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology*, 108(1): 40-50.

Reinecker HC, MacDermott RP, Mirau S, Dignass A, and Podolsky DK (1996) Intestinal epithelial cells both express and respond to interleukin 15. *Gastroenterology*, 111(6): 1706-1713.

Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, and Axon ATR (1999) Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet*, 354(9179): 635-639.

Resta-Lenert S and Barrett KE (2003) Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). *Gut*, 52(7): 988-997.

Riesen FK, Rothen-Rutishauser B, and Wunderli-Allenspach H (2002) A ZO1-GFP fusion protein to study the dynamics of tight junctions in living cells. *Histochem Cell Biol*, 117(4): 307-315.

Roberfroid MB, Bornet F, Bouley C, and Cummings JH (1995) Colonic microflora: nutrition and health. Summary and conclusions of an International Life Sciences Institute (ILSI) workshop held in Barcelona, Spain. *Nutr Rev*, 53(5): 127-130.

Roe AJ, Hoey DE, and Gally DL (2003) Regulation, secretion and activity of type III-secreted proteins of enterohaemorrhagic *Escherichia coli* O157. *Biochem Soc Trans*, 31(Pt 1): 98-103.

Rogler G (2004) Update in inflammatory bowel disease pathogenesis. *Curr Opin Gastroenterol*, 20(4): 311-317.

Rogler G, Daig R, Aschenbrenner E, Vogl D, Schlottmann K, Falk W, Gross V, Scholmerich J, and Andus T (1998) Establishment of long-term primary cultures of human small and large intestinal epithelial cells. *Lab Invest*, 78(7): 889-890.

Rosenfeldt V, Michaelsen KF, Jakobsen M, Larsen CN, Moller PL, Pedersen P, Tvede M, Weyrehter H, Valerius NH, and Paerregaard A (2002) Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. *Pediatr Infect Dis J*, 21(5): 411-416.

Russell RK, Drummond HE, Nimmo EE, Anderson N, Smith L, Wilson DC, Gillett PM, McGrogan P, Hassan K, Weaver LT, Bisset M, Mahdi G, and Satsangi J (2005) Genotype-phenotype analysis in childhood-onset Crohn's disease: NOD2/CARDI 5 variants consistently predict phenotypic characteristics of severe disease. *Inflamm Bowel Dis*, 11(11): 955-964.

Rutledge BJ, Rayburn H, Rosenberg R, North RJ, Gladue RP, Corless CL, and Rollins BJ (1995) High level monocyte chemoattractant protein-1 expression in transgenic mice increases their susceptibility to intracellular pathogens. *J Immunol*, 155(10): 4838-4843.

Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, Takano H, Noda T, and Tsukita S (1998) Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J Cell Biol*, 141(2): 397-408.

Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, and Tsukita S (2000) Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell*, 11(12): 4131-4142.

Sakaguchi T, Kohler H, Gu XB, McCormick BA, and Reinecker HC (2002) *Shigella flexneri* regulates tight junction-associated proteins in human intestinal epithelial cells. *Cell Microbiol*, 4(6): 367-381.

Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, and Rowland I (1998) Functional food science and gastrointestinal physiology and function. *Br J Nutr*, 80 (Suppl 1): 147-171.

Sansonetti P (2002) Host-pathogen interactions: the seduction of molecular cross talk. *Gut*, 3 (Suppl.): III2-III8.

Sansonetti PJ (2004) War and peace at mucosal surfaces. *Nat Rev Immunol*, 4(12): 953-964.

Sartor RB (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: Antibiotics, probiotics, and prebiotics. *Gastroenterology*, 126(6): 1620-1633.

Saxelin M, Tynkkynen S, Mattila-Sandholm T, and de Vos WM (2005) Probiotic and other functional microbes: from markets to mechanisms. *Curr Opin Biotechnol*, 16(2): 204-211.

Schaefer TM, Desouza K, Fahey JV, Beagley KW, and Wira CR (2004) Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. *Immunology*, 112(3): 428-436.

Schmauder-Chock EA, Chock SP, and Patchen ML (1994) Ultrastructural localization of tumour necrosis factor-alpha. *Histochem J*, 26(2): 142-151.

Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, Riecken EO, and Schulzke JD (1999) Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology*, 116(2): 301-309.

Schultz M, Scholmerich J, and Rath HC (2003) Rationale for probiotic and antibiotic treatment strategies in inflammatory bowel diseases. *Dig Dis*, 21(2): 105-128.

Schultz M, Strauch UG, Linde HJ, Watzl S, Obermeier F, Gottl C, Dunger N, Grunwald N, Scholmerich J, and Rath HC (2004) Preventive effects of *Escherichia coli* strain Nissle 1917 on acute and chronic intestinal inflammation in two different murine models of colitis. *Clin Diagn Lab Immunol*, 11(2): 372-378.

Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO, and Fromm M (1998) Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res*, 43(4 Pt 1): 435-441.

Senok AC, Ismaeel AY, and Botta GA (2005) Probiotics: facts and myths. *Clin Microbiol Infect*, 11(12): 958-966.

Sherman PM, Johnson-Henry KC, Yeung HP, Ngo PSC, Goulet J, and Tompkins TA (2005) Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7 and enteropathogenic *E. coli* O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. *Infect Immun*, 73(8): 5183-5188.

Shibahara T, Wilcox JN, Couse T, and Madara JL (2001) Characterization of epithelial chemoattractants for human intestinal intraepithelial lymphocytes. *Gastroenterology*, 120(1): 60-70.

Shifflett DE, Clayburgh DR, Koutsouris A, Turner JR, and Hecht GA (2005) Enteropathogenic *E.coli* disrupts tight junction barrier function and structure in vivo. *Lab Invest*, 85(10): 1308-1324.

Simon GL and Gorbach SL (1984) Intestinal flora in health and disease. *Gastroenterology*, 86(1): 174-193.

Simonovic I, Rosenberg J, Koutsouris A, and Hecht G (2000) Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. *Cell Microbiol*, 2(4): 305-315.

St Amant DC, Valentin-Bon IE, and Jerse AE (2002) Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect Immun*, 70(12): 7169-7171.

Staehelin LA (1973) Further observations on the fine structure of freeze-cleaved tight junctions. *J Cell Sci*, 13(3): 763-786.

Standring S (Ed.) (2005), Gray's anatomy, 39th edition, Churchill Livingstone, 2005.

Stephen AM and Cummings JH (1980) The microbial contribution to human faecal mass. *J Med Microbiol*, 13(1): 45-56.

Stevenson BR, Siliciano JD, Mooseker MS, and Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol*, 103(3): 755-766.

Sturm A, Rilling K, Baumgart DC, Gargas K, bou-Ghazale T, Raupach B, Eckert J, Schumann RR, Enders C, Sonnenborn U, Wiedenmann B, and Dignass AU (2005) *Escherichia coli* Nissle 1917 distinctively modulates T-cell cycling and expansion via toll-like receptor 2 signaling. *Infect Immun*, 73(3): 1452-1465.

Sullivan A and Nord CE (2005) Probiotics and gastrointestinal diseases. *J Intern Med*, 257(1): 78-92.

Sun J, Gunzer F, Westendorf AM, Buer J, Scharfe M, Gößling F, Blöcker H, and Zeng AP (2005) Genomic peculiarity of coding sequences and metabolic potential of probiotic *Escherichia coli* strain Nissle 1917 inferred from raw genome data. *J Biotechnol*, 117(2): 147-161.

Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, and Lochs H (2002) Mucosal flora in inflammatory bowel disease. *Gastroenterology*, 122(1): 44-54.

Swidsinski A, Schlien P, Pernthaler A, Gottschalk U, Barlehner E, Decker G, Swidsinski S, Strassburg J, Loening-Baucke V, Hoffmann U, Seehofer D, Hale LP, and Lochs H (2005) Bacterial biofilm within diseased pancreatic and biliary tracts. *Gut*, 54(3): 388-395.

Taha Y, Raab Y, Larsson A, Carlson M, Loof L, Gerdin B, and Thorn M (2004) Vascular endothelial growth factor (VEGF) - A possible mediator of inflammation and mucosal permeability in patients with collagenous colitis. *Dig Dis Sci*, 49(1): 109-115.

Takeda K and Akira S (2004) Microbial recognition by Toll-like receptors. *J Dermatol Sci*, 34(2): 73-82.

Takeda K and Akira S (2005) Toll-like receptors in innate immunity. *Int Immunol*, 17(1): 1-14.

Tannock GW (2001) Molecular assessment of intestinal microflora. *Am J Clin Nutr*, 73(Suppl. 2): 410-414.

Taub DD, Sayers TJ, Carter CR, and Ortaldo JR (1995) Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol*, 155(8): 3877-3888.

Taylor V and Suter U (1996) Epithelial membrane protein-2 and epithelial membrane protein-3: Two novel members of the peripheral myelin protein 22 gene family. *Gene*, 175(1-2): 115-120.

Thompson GR and Trexler PC (1971) Gastrointestinal structure and function in germ-free or gnotobiotic animals. *Gut*, 12(3):230-235.

Tissier H (1906) Traitement des infections intestinales par la méthode de la flore bactérienne de l'intestin. *CR Soc Biol*, 60: 359-361.

Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, Williams BR, Major J, Hamilton TA, Fenton MJ, and Vogel SN (2002) TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol*, 3(4): 392-398.

Tsukamoto T and Nigam SK (1997) Tight junction proteins form large complexes and associate with the cytoskeleton in an ATP depletion model for reversible junction assembly. *J Biol Chem*, 272(26): 16133-16139.

Tsukita S, Furuse M, and Itoh M (2001) Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol*, 2(4): 285-293.

Turner JR, Black ED, Ward J, Tse CM, Uchwat FA, Alli HA, Donowitz M, Madara JL, and Angle JM (2000) Transepithelial resistance can be regulated by the intestinal brush-border Na+/H+ exchanger NHE3. *Am J Physiol*, 279(6): 1918-1924.

Turner JR, Rill BK, Carlson SL, Carnes D, Kerner R, Mrsny RJ, and Madara JL (1997) Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am J Physiol*, 42(4): 1378-1385.

Tursi A, Brandimarte G, Giorgetti GM, Forti G, Modeo ME, and Gigliobianco A (2004) Low-dose balsalazide plus a high-potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acute mild-to-moderate ulcerative colitis. *Med Sci Monit*, 10(11): PI126-131.

Umesaki Y, Setoyama H, Matsumoto S, and Okada Y (1993) Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. *Immunology*, 79(1):32-37.

Van Belzen N, Dinjens WN, Diesveld MP, Groen NA, van der Made AC, Nozawa Y, Vlietstra R, Trapman J, and Bosman FT (1997) A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. *Lab Invest*, 77(1): 85-92.

Van Coillie E, Van Damme J, and Opdenakker G (1999) The MCP eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev*, 10(1): 61-86.

Van Itallie CM and Anderson JM (1999) Tight-junction protein ZO-1 isoforms (alpha(+) and alpha(-)) show differential extractability and epidermal-growth-factor-induced tyrosine phosphorylation in A431 cells. *Protoplasma*, 206(4): 211-218.

Van Niel G, Mallegol J, Bevilacqua C, Candalh C, Brugiere S, Tomaskovic-Crook E, Heath JK, Cerf-Bensussan N, and Heyman M (2003) Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut*, 52(12): 1690-1697.

Vantrappen GR and Peters TL (1974) Proceedings: The production of lysozyme by the Paneth cell. *Gut*, 15(10): 86-87.

Veckman V, Miettinen M, Matikainen S, Lande R, Giacomini E, Coccia EM, and Julkunen I (2003) Lactobacilli and streptococci induce inflammatory chemokine production in human macrophages that stimulates Th1 cell chemotaxis. *J Leukoc Biol*, 74(3): 395-402.

Venturi A, Gionchetti P, Rizzello F, Johansson R, Zucconi E, Brigidi P, Matteuzzi D, and Campieri M (1999) Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol Ther*, 13(8): 1103-1108.

Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, and Robinson C (1999) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest*, 104(1): 123-133.

Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, and Turner JR (2005) Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am J Pathol*, 166(2): 489-519.

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, Schroder JM, and Stange EF (2004) NF-kappa B- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: A novel effect of a probiotic bacterium. *Infect Immun*, 72(10): 5750-5758.

Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, Shen B, Schaeffeler E, Schwab M, Linzmeier R, Feathers RW, Chu H, Lima H, Jr., Fellermann K, Ganz T, Stange EF, and Bevins CL (2005) Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A*, 102(50): 18129-18134.

Wertz IE, O'Rourke KM, Zhou HL, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, and Dixit VM (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappa B signalling. *Nature*, 430(7000): 694-699.

Westendorf AM, Gunzer F, Deppenmeier S, Tapadar D, Hunger JK, Schmidt MA, Buer J, and Bruder D (2005) Intestinal immunity of *Escherichia coli* NISSLE 1917: a safe carrier for therapeutic molecules. *Fems Immunol Med Microbiol*, 43(3): 373-384.

Wilker EW, Grant RA, Artim SC, and Yaffe MB (2005) A structural basis for 14-3-3 sigma functional specificity. *J Biol Chem*, 280(19): 18891-18898.

Willott E, Balda MS, Heintzelman M, Jameson B, and Anderson JM (1992) Localization and differential expression of two isoforms of the tight junction protein ZO-1. *Am J Physiol*, 262(5 Pt 1):1119-1124.

Winsor GL, Waterhouse CC, MacLellan RL, and Stadnyk AW (2000) Interleukin-4 and IFN-gamma differentially stimulate macrophage chemoattractant protein-1 (MCP-1) and eotaxin production by intestinal epithelial cells. *J Interferon Cytokine Res*, 20(3): 299-308.

Wirtz S and Neurath MF (2000) Animal models of intestinal inflammation: new insights into the molecular pathogenesis and immunotherapy of inflammatory bowel disease. *Int J Colorectal Dis*, 15(3): 144-160.

Wolpe SD, Davatelis G, Sherry B, Beutler B, Hesse DG, Nguyen HT, Moldawer LL, Nathan CF, Lowry SF, and Cerami A (1988) Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp Med*, 167(2): 570-581.

Worthington RE, Carroll RC, and Boucheix C (1990) Platelet activation by CD9 monoclonal antibodies is mediated by the Fc gamma II receptor. *Br J Haematol*, 74(2): 216-22.

Wu S, Lim KC, Huang J, Saidi RF, and Sears CL (1998) *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci U S A*, 95(25): 14979-14984.

Wu Z, Nybom P, and Magnusson KE (2000) Distinct effects of *Vibrio cholerae* haemagglutinin/protease on the structure and localization of the tight junction-associated proteins occludin and ZO-1. *Cell Microbiol*, 2(1): 11-17.

Xavier RJ and Podolsky DK (2000) Microbiology. How to get along-friendly microbes in a hostile world. *Science*, 289(5484): 1483-1484.

Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV, and Gordon JI (2003) A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science*, 299(5615): 2074-2076.

Yamagami H, Yamagami S, Inoki T, Amano S, and Miyata K (2003) The effects of proinflammatory cytolkines on cytokine-chemokine gene expression profiles in the human corneal endothelium. *Invest Ophthalmol Vis Sci*, 44(2): 514-520.

Yio XY and Mayer L (1997) Characterization of a 180-kDa intestinal epithelial cell membrane glycoprotein, gp180 - A candidate molecule mediating T cell epithelial cell interactions. *J Biol Chem*, 272(19): 12786-12792.

Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, and Leonard EJ (1989) Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *Febs Lett*, 244(2): 487-493.

Yuhan R, Koutsouris A, Savkovic SD, and Hecht G (1997) Enteropathogenic *Escherichia coli*-induced myosin light chain phosphorylation alters intestinal epithelial permeability. *Gastroenterology*, 113(6): 1873-1882.

Zahraoui A, Joberty G, Arpin M, Fontaine JJ, Hellio R, Tavitian A, and Louvard D (1994) A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. *J Cell Biol*, 124(1-2): 101-115.

Zhang Y, Emmanuel N, Kamboj G, Chen J, Shurafa M, Van Dyke DL, Wiktor A, and Rowley JD (2004) PRDX4, a member of the peroxiredoxin family, is fused to AML1 (RUNX1) in an acute myeloid leukemia patient with a t(X;21)(p22;q22). *Genes Chromosomes Cancer*, 40(4): 365-70.

Danksagung

Die vorliegende Arbeit wurde an der Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, in der Abteilung Zellbiologie und Immunologie, Arbeitsgruppe Mukosale Immunität sowie der Medizinischen Hochschule Hannover, Institut für Medizinische Mikrobiologie und Krankenhaushygiene angefertigt.

Ich danke dem Mentor dieser Arbeit, Herrn Prof. Dr. J. Wehland für die Übernahme des Hauptreferats, Frau Prof. Dr. P. Dersch für die Übernahme des Korreferats und Herrn Prof. Dr. S. Dübel für die Bereitschaft als Prüfer zur Verfügung zu stehen. Herrn Prof. Dr. S. Suerbaum danke ich für aufschlußreiche Diskussionen meiner Daten.

Mein besonderer Dank gilt Herrn PD Dr. med. F. Gunzer für die interessante Themenstellung und sein Vertrauen in meine Fähigkeiten. Er gab mir die Möglichkeit, mich frei zu entfalten und meine Ideen erfolgreich umzusetzen. Herrn Prof. Dr. med. J. Buer bin ich sehr dankbar für sein Engagement und die großzügige Bereitstellung aller benötigten Mittel. Desweiteren danke ich ihm für die Möglichkeit, meine Dissertation in seiner Arbeitsgruppe anzufertigen.

Mein herzlicher Dank geht an Dr. Astrid M. Westendorf für ihr unermüdliches Engagement und ihre Gedankenanstöße. Ich danke Dir für Deine Unterstützung und Deine konstruktive Kritik, die mich immer ein Stück weiter brachte. Dir, Dr. Wiebke Hansen und Dr. Dunja Bruder danke ich für Eure Hilfe bei der Bewältigung der großen und kleinen Hürden des Laboralltags. Ich konnte mich jederzeit an Euch wenden. Danke!

Bei allen Mitarbeitern der AG Mukosale Immunität möchte ich mich für das familiäre, immer hilfsbereite und herzliche Arbeitsklima bedanken. Insbesondere danke ich Silvia Prettin, die mir einige Male hilfreich zur Seite sprang. Ihr sowie Ulrike Gölden, Marcus Gereke und Simone Reinwald danke ich für die tolle gemeinsame Zeit.

Desweiteren danke ich der AG von PD Dr. med. Gunzer für die anregenden Diskussionen und die freundliche Atmosphäre sowie Dr. med. vet. André Bleich, Marco Metzger und Dr. Manfred Rohde für erfolgreiche Kooperationen.

Ich möchte an dieser Stelle Dr. Jo Lauber gedenken, der mich in den ersten Monaten meiner Doktorarbeit im Labor begleitet hat. Er wird mir immer in guter Erinnerung bleiben.

Ganz besonders bedanke ich mich bei meinen Eltern, meiner Schwester Seyda und Marco, die immer für mich da sind, fest an mich und meine Fähigkeiten glauben und mit mir Erfolge aber auch Niederschläge teilen.